Yeast orthologue of Niemann-Pick C1 protein in oxidative stress resistance and mitochondrial function

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General Abbreviations

ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
CFU	Colony forming units
CNS	Central nervous system
CoA	Coenzyme A
CoQ10	Coenzyme Q10
CLS	Chronological lifespan
DHE	Dihydroethidium
DHS	Dihydrosphingosine
DNA	Deoxyribonucleic acid
DNP	2,4 – dinitrophenylhydrazone
DNPH	2,4 - dinitrophenylhydrazine
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
GalNac transferase	N-acetylgalactosaminyl-transferases
GM2;GM3	Gangliosides
GSH	Glutathione
GSL	Glycosphingolipid
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
LCB	Long chain sphingoid base

LDL	Low density lipoprotein
LE/Lys	Late endosome/lysosome
LMW	Low molecular weight
MD	Menadione
MDA	Malondialdehyde
mtDNA	Mitochondrial Deoxyribonucleic acid
NPC	Niemann-Pick type C
$O_2^{\bullet-}$	Superoxide anion
OD	Optical density
$OH^{\bullet}$	Hydroxyl radical
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHS	Phytosphingosine
PKB	Protein kinase B
PKC	Protein kinase C
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
ROS	Reactive oxygen species
SC	Synthetic complete
SD	Standard deviation
SDS – PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM	Sphingomyelin
SPT	Serine palmitoyltransferase
SSD	Sterol sensing domain

TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TOR	Target of rapamycin
TPBS	Phosphate buffered saline plus tween
UK	United Kingdom
UV	Ultra-violet
YPD	Yeast peptone dextrose

Abstract

Sphingolipid storage diseases are a group of disorders, caused by defects in lysosomal hydrolytic processes or lipid transport that lead to the accumulation of lipids in the endosomal-lysosomal system. Among this group are the Niemann-Pick disorders. The Niemann-Pick type C (NPC) is characterized by a progressive neurodegenerative phenotype and is caused by mutations in NPC1 (95% of the cases) or NPC2 (5% of the cases). Both proteins seem to regulate intracellular lipid transport between lysosomes and endosomes. It has been suggested that alterations in this transport compromises mitochondrial activity.

The yeast *Saccharomyces cerevisiae* Ncr1p is an orthologue of human NPC1 protein. It seems to be involved in ergosterol trafficking and can functionally complement the loss of human NPC1, suppressing lipid trafficking defects in NPC cells. Yeast cells deleted for *NCR1* have been used as a eukaryotic model system to characterize conserved cellular mechanisms associated with NPC1 disease.

This work aims to uncover the role of Ncr1p in oxidative stress resistance and mitochondrial function and to characterize changes in cell signalling pathways associated with Δ*ncr1* phenotype. Wild type and Δ*ncr1* mutant cells were treated with hydrogen peroxide or menadione, a superoxide generating drug, and cellular viability was determined and correlated with oxidative stress markers, namely protein carbonylation, levels of reactive oxygen species and lipid peroxidation. The results show that Δ*ncr1* cells exhibited a significant decrease in hydrogen peroxide resistance, compared to the observed in wild type cells, associated with an increase of oxidative stress markers. However, menadione resistance was not affected in the mutant strain.

Moreover, Ncr1p deficiency was associated with loss of mitochondrial function, as shown by the incapacity of this mutant to grow on glycerol medium, a non-fermentable carbon source. Mitochondrial function is important for oxidative stress resistance and is regulated by protein kinases and phosphatases, such as Tor1p, Sit4p and Sch9p. Tor1p is the phosphatidylinositol-related protein kinase subunit of the rapamycin-sensitive TORC1 complex that controls cell growth in response to nutrient availability and cellular stresses. Sit4p is a serine-threonine ceramide-activated protein phosphatase related to type 2A family of protein phosphatases (PP2A) that are involved in cell cycle regulation. Sch9p is a serine-threonine kinase with possible homology to mammalian ribosomal S6 kinase (S6K) or Protein Kinase B (PKB/AKT) that is activated by TORC1 or phytosphingosine through Pkh1/2p pathway and is involved in the regulation of glucose metabolism. Notably, the hydrogen peroxide sensitivity and the growth defect on glycerol displayed by

Δ*ncr1* cells were suppressed by deletion of *SIT4 and SCH9* but not by *TOR1.* The oxidative stress resistance of Δ*ncr1*Δ*sit4* and Δ*ncr1*Δ*sch9* double mutants was associated with lower levels of reactive oxygen species.

These results suggest that Ncr1p regulate cellular processes important for mitochondrial function and hydrogen peroxide resistance through modulation if Sit4p and Sch9p signalling pathways.

Resumo

As doenças de armazenamento de esfingolípidos constituem um grupo de doenças causadas por defeitos em processos lisossomais ou transporte lipídico, originando a acumulação de lípidos no sistema endossomal-lisossomal. Entre este grupo encontra-se a doença de Niemann-Pick. A doença de Niemann-Pick do tipo C (NPC) é caracterizada por um fenótipo neurodegenerativo progressivo e é causada por mutações em *NPC1* (95 % dos casos) ou *NPC2* (5 % dos casos). Estas proteínas parecem estar envolvidas na regulação do transporte lipídico intracelular entre lisossomas e endossomas.

Tem sido sugerido que alterações neste transporte comprometem a actividade da mitocôndria. A levedura *Saccharomyces cerevisiae* possui uma proteína ortóloga à NPC1 humana que se designa por Ncr1p. Esta proteína parece estar envolvida no transporte de ergosterol em levedura e consegue complementar funcionalmente a perda de NPC1 humana, suprimindo os defeitos no transporte lipídico em células de NPC. As leveduras com deleção do gene *NCR1* tem sido usadas para caracterizarem mecanismos celulares conservados associados a doença de NPC1.

Este trabalho tem como objectivo estudar o papel da proteína Ncr1p na resistência ao stress oxidativo e disfunções mitocondriais, bem como, caracterizar alterações em vias de sinalização associadas com o fenótipo das células Δ*ncr1*. A estirpe selvagem e respectivos mutantes Δ*ncr1* foram tratadas com peróxido de hidrogénio ou menadiona (utilizada para induzir formação de anião superóxido) e a viabilidade celular foi correlacionado com marcadores de stress oxidativo, tais como, níveis de espécies reactivas de oxigénio intracelulares, peroxidação lipídica e carbonilação de proteínas. Os resultados demonstram uma diminuição significativa da resistência ao peróxido de hidrogénio por parte do mutante Δ*ncr1* comparado com o observado na estirpe selvagem, associado com o aumento dos marcadores de stress oxidativo. Contudo, a resistência a menadiona não foi afectada na estirpe mutante.

Além disso, a deficiência em Ncr1p foi relacionada com perda da função mitocondrial, como demonstrado pela incapacidade do mutante crescer em meio com glicerol (uma fonte de carbono não fermentável). A função mitocondrial é importante para a resistência ao stress oxidativo e é regulada por proteínas cinases e fosfatases tais como Tor1p, Sit4p e Sch9p. Tor1p é uma proteína cinase, subunidade do complexo TORC1 (sensível a rapamicina) que controla o crescimento celular em resposta a nutrientes e stress celular. Sit4p é uma serina- treonina fosfatase activada por ceramida e relacionada com a família das proteínas fosfatases do tipo 2A (PP2A) que esta envolvida

na regulação do ciclo celular. Sch9p é uma serina-treonina cinase que tem possível homologia com a cinase ribossomal S6 (S6K) ou a proteína cinase B (PKB/AKT) dos mamíferos. Esta proteína é directamente activada pelo TORC1 ou por fitoesfingosina através da via do Pkh1/2p e está envolvida na regulação do metabolismo da glucose. Curiosamente a deleção dos genes *SIT4* e *SCH9* suprimiram a sensibilidade ao peróxido de hidrogénio bem como os defeitos no crescimento em glicerol exibidos pelas células Δ*ncr1*, mas o mesmo não se verificou com a deleção do *TOR1*. A resistência ao stress oxidativo dos duplos mutantes Δ*ncr1*Δ*sit4* e Δ*ncr1*Δ*sch9*.foi associada com níveis diminuídos de espécies reactivas de oxigénio.

Estes resultados sugerem que a proteína Ncr1p regula processos celulares importantes para a função mitocondrial e resistência ao peróxido de hidrogénio através da modulação das vias de sinalização do Sit4p e Sch9p.

Chapter 1

Introduction

1. Niemann-Pick disease

Sphingolipid-storage diseases are a group of genetic disorders triggered by deficiencies in lysosomal hydrolytic activities or lipid transport that results in the accumulation of lipids (e.g., cholesterol) in the endossomal-lysosomal network [1]. Among this group is Niemann-Pick. Albert Niemann and Ludwig Pick were the pioneers describing this disease in 1920. The term “Niemann-Pick disease” has been used to designate a heterogeneous group of autosomal recessive lysosomal disorders, with common types of hepatosplenomegaly and sphingomyelin storage in reticuloendothelial and parenchymal tissue, with or without neurological involvement [2]. In 1958, Crocker and Farber demonstrated that there was a broad variability in age of onset, clinical expression and in the level of sphingomyelin storage in tissues, which led to a classification of this disorder into four subgroups: A to D [3]. Patients with type A exhibited a severe neurovisceral disease, while type B had a chronic course with visceral contribution only. The accumulation of sphingomyelin was found in those patients [3], and later work demonstrated a loss of efficiency in acidic sphingomyelinase allowing the classification of type A and B as sphingomyelin storage disorders [4]. These results were not verified in patients with Niemann-Pick type C and D, which have a sub-acute nervous system involvement, with moderate/slower course and a mild visceral storage. Type D patients were individualized essentially on their homogenous Nova Scotia Acadian origin (allelic to type C). Later work led to a reclassification of type C as a cellular lipid trafficking disorder [5].

2. Niemann-Pick Type C (NPC)

Niemann-Pick type C (NPC) designates disorders characterized by unique abnormalities of intracellular transport of endocytosed cholesterol with accumulation of unesterified cholesterol in lysosomes, late endosomes and Golgi complex [2,6]. Besides cholesterol sequestration, NPC cells accumulate other lipids in particular sphingomyelin (SM), glycosphingolipids (GSLs) and sphingosine [7–11]. NPC is related to a progressive neurodegenerative phenotype and in most cases is fatal [12].

Advances in the knowledge of the disease led to the description of two genetic complementation groups and the subsequent characterization of the two genes: NPC1 and NPC2. They are represented in different proportions in the population – NPC1 is involved in 95% of the cases [12], while NPC2 is related to rare cases [2]. NPC is caused

by loss-of-function point mutations in either NPC1 or NPC2 proteins, which mediate proper intracellular lipid transport through pathways that remain unclear [1].

2.1. Epidemiology

The prevalence of NPC is difficult to estimate, not only because of the insufficient clinical awareness and difficult diagnosis but also to a relative difficulty of biochemical testing. The prevalence in Western Europe (France, UK, and Germany) has been estimated to be around 0.66 to 0.83 births per 100.000. In Australia, Netherland and Portugal the prevalence is 0.45, 0.37 and 2.2 per 100.000, respectively. The low prevalence in some countries may be explained by a lack of diagnosis in the years of birth and the fact that the clinical spectrum was only recognized in the early 1990s [2,13,14].

2.2. Clinical description and diagnosis

NPC has an extremely heterogeneous clinical presentation characterized by a wide variety of symptoms that are not specific to the disease and with an age of onset fluctuating from perinatal period until adult age [2,15]. The lifespan of the patients varies between a few days until over 60 years of age [16,17], and there is no exact correlation between disease-causing mutations and severity of clinical phenotype [18].

Classically, NPC is a neurovisceral condition. This disease affects internal organs (liver and spleen) and the first symptoms usually described are hepatosplenomegaly (that seems to be variable and decrease with time) or obstructive jaundice. Except for the perinatal period, the systemic disease is usually not very severe and well tolerated. Apart from a small percentage of patients that die at birth, or in the first 6 months of life, and exceptional adult cases, all patients develop a progressive and fatal neurological disease [1,2].

This disease can be classified into four major groups according to the onset of neurological symptoms: early infantile, late infantile, juvenile, and adult forms [18]. However, the classic form of NPC, which accounts for 60-70 % of the cases, appears between the ages of 3 and 15 years [1].The age at arrival of neurological symptoms has a major influence on disease progression. If neurological symptoms arise early in life, the rate of deterioration is generally faster and premature death occurs sooner [15]. The

neurological symptoms can be very diverse, according to the age of onset of the disease and are resumed in the Figure 1.1.

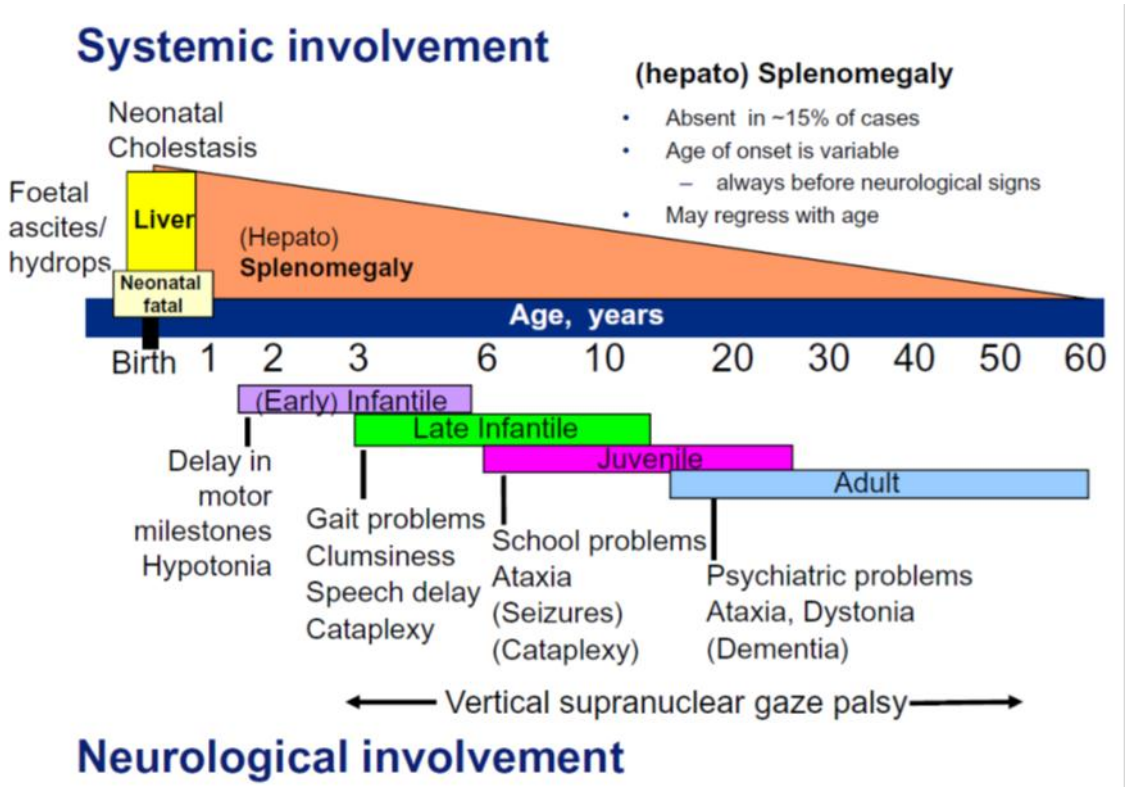


Figure 1.1- Niemann-Pick disease type C as a neurovisceral disease. Adapted from [2]

The heterogeneity of the clinical manifestations of NPC disease is not totally detected when it comes to the biochemical level of the disease. The majority of cases present prominent accumulations of unesterified cholesterol, sphingolipids and complex gangliosides in late endosomes and lysosomes, but a subset of patients with specific mutations reveals less lipid storage [19]. The evolution of NPC in the peripheral tissues is characterised by an enlargement of the liver and the spleen that results from the presence of lipid-laden macrophages. Kupffer cells (resident macrophages) in liver and splenic macrophages exhibit a clear cytoplasmic vacuolization that results from the accumulation of cholesterol, phospholipids and glycolipids [1]. Impairment of lipid trafficking also has severe consequences in the central nervous system (CNS), leading to neuron loss throughout the brain [20]. Similarly to other neurodegenerative diseases, such as Alzheimer disease, NPC cells accumulate intracellular aggregates of microtubule-binding protein tau [21].

Because NPC is a very heterogeneous disease, the correct diagnosis is often very difficult to achieve. Even after multiple clinical screening tests to distinguish NPC from other neurological diseases, the validation of the diagnosis requires also biochemical and molecular-genetic laboratory testing. The key laboratory diagnostic test for NPC is filipin staining of cultured skin fibroblasts from patients, to detect the accumulation of free cholesterol in lysosomes, and a biochemical approach, to monitor defective cholesterol esterification in low density lipoprotein (LDL)-challenged cells [22]. Finally, a molecular genetic test for NPC1 and NPC2 is essential to a prenatal diagnosis, and to confirm diagnosis in patients with a different biochemical phenotype [15,22]. Although there are therapies for some of the clinical manifestations, currently there are no effective treatments available to patients with this disorder [1].

2.3. NPC1 and NPC2

NPC disease is genetically heterogeneous, and it is possible to distinguish two complementation groups: NPC1 and NPC2. NPC1, the major group for this disease, was mapped to the proximal long arm of chromosome 18, and mutations in NPC1 were found in approximately 95 % of the patients with NPC [23]. NPC2 was mapped to the long arm of chromosome 14 and mutations in NPC2 were found in a minor group of patients (5%) [24].

The NPC1 gene is localized in chromosome 18q11-q12 and translates a 1278 amino acid membrane protein with 13 transmembrane domains, which is localised in late endosomes and interact with lysosomes and the trans-Golgi network [25,26]. This protein has numerous domains that are preserved among species. The sterol sensing domain (SSD) is localized in the region between amino acids 615 and 797, and display high homology to SSD of other integral membrane proteins that act in response to endoplasmic reticulum (ER) cholesterol (HMG-CoA reductase) [27].The NPC1 domain (amino acids 55-165) is localized in the N-terminal loop and contains a leucine zipper motif (amino acids 73-94). Also, there is a large cysteine-rich loop found between amino acids 855-1098, which contains a ring-finger motif and is a likely site for protein-protein interactions (Figure 1.2) [28].

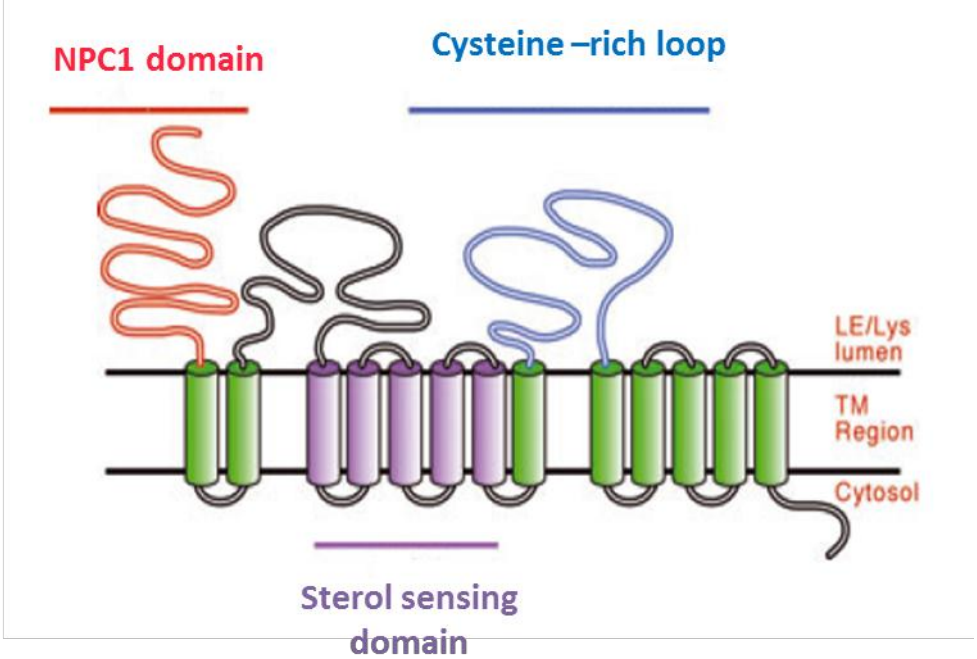


Figure 1.2- Topology of NPC1. LE/Lys, late endosomes/lysosomes. TM, transmembrane region. Adapted from [11].

The number of mutations that cause NPC1 disease is approximately 300, with a large majority of missense mutations, and more than 60 polymorphisms have been described in NPC1 gene [2]. The missense mutations are spread throughout the NPC1 gene and influence the functional domains, except the leucine zipper motif. More than one-third of the mutations are localized in the cysteine-rich loop, and there is a hot spot that contain three of the most frequent mutations, localised between amino acids 927 and 958 [28]. The most frequent mutation is in the allele encoding p.I1061T, is related with prominent disturbs in fibroblasts cholesterol trafficking of patients and with a juvenile neurological onset of NPC [29]. There are few studies on genotype-phenotype relations but, the NPC1 patients generally present a good correlation between nonsense or frameshift mutations and the most severe neurological course. The SSD (along with cysteine-rich luminal loop) also have mutations that highlighted the significant function of both domains. Homozygous mutations in SSD seem to be very harmful, corresponding to a lack of mature NPC1 protein and to a very severe phenotype at both clinical and biochemical levels [30]. The distribution of the mutations in the NPC1 protein is illustrated in the Figure 1.3.

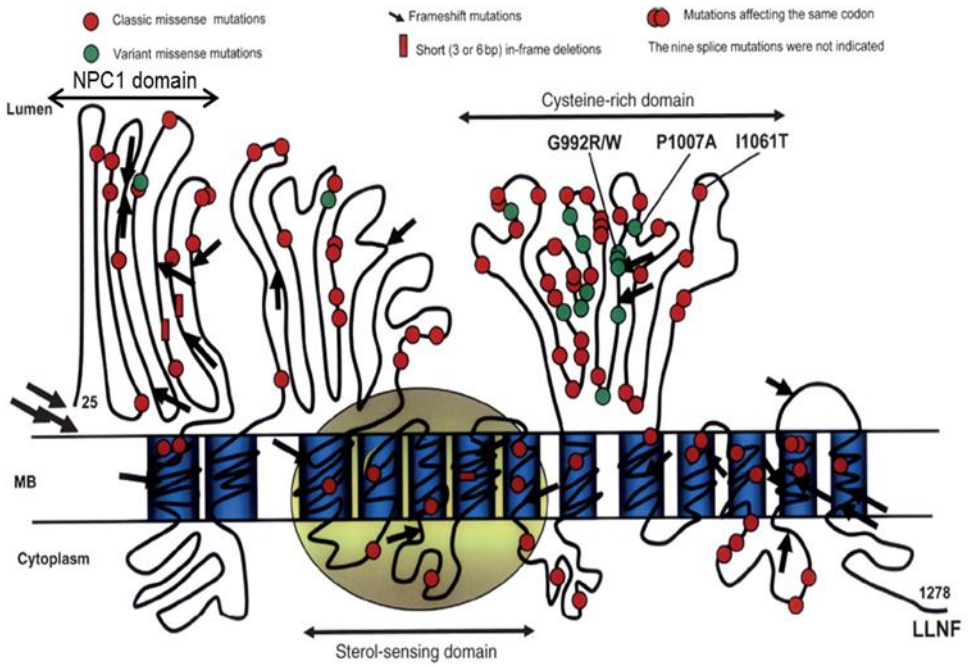


Figure 1.3 - Topology of NPC1 mutations. Adapted from [28].

NPC2 encodes a small (132 amino acids) and soluble lysosomal protein. This proteins is transported to the lysosome via the mannose-6-phosphate receptor and binds to cholesterol with high affinity [31]. Mutations in this protein are associated with very severe clinical phenotypes [19,32]. The mature form of NPC2 is a glycoprotein expressed abundantly in several tissues [24]. Studies show that NPC2 has high affinity to cholesterol and identified a hydrophobic cholesterol-binding pocket [33]. There are few cases of NPC2 disease, but all of them show remarkable abnormalities of cellular cholesterol processing. It was suggested that NPC1 could be a regulator of NPC2 transport, but it was not confirmed. With the increase of NPC2 cases, it is clear that it has also high heterogeneity as observed for NPC1 [28].

The exact functions of NPC1 and NPC2 proteins have not yet been fully elucidated. Regarding the function of NPC1, one study based on its homology with prokaryotic permeases of the resistance-nodulation-division (RND) family showed that NPC1 may function as a transmembrane molecular pump, which acts as a permease transporting fatty acids across cellular membrane in *Escherichia coli* [27]. Despite some ambiguous results, some experimental data suggest that NPC1 plays a role in regulation or mediation of retrograde transport of lysosomal cargo in the late endossomal-lysosomal pathway [26]. The majority of cell biological studies regarding NPC pathology are referent to cells defective only in NPC1. In fact, NPC2 patients are rare and the models of study

are scarce. NPC1 has several models of work, such as tissue samples from affected patients, cells from NPC1$^{-/-}$ mice [34] or several cell lines in which NPC1 has been mutated [1].

There are strong evidences that the NPC1 and NPC2 proteins function in the same pathway, because mutations in both genes induce a similar phenotype. Furthermore, genetic studies reveal that NPC2 alone is not sufficient for cholesterol egress from lysosomes, and hence it requires NPC1 [35]. Based on this knowledge, some models were described to try to explain how these proteins cooperate. One example proposed by Kown and associates is illustrated in figure 1.4. In this model, cholesterol released within the lysosome binds to NPC2 with its hydroxyl group exposed. Then, cholesterol is transfered to the N-terminal domain of NPC1, reversing its orientation, so that the hydrophobic side chain could lead the way into the membrane and/or the glycocalix [35].

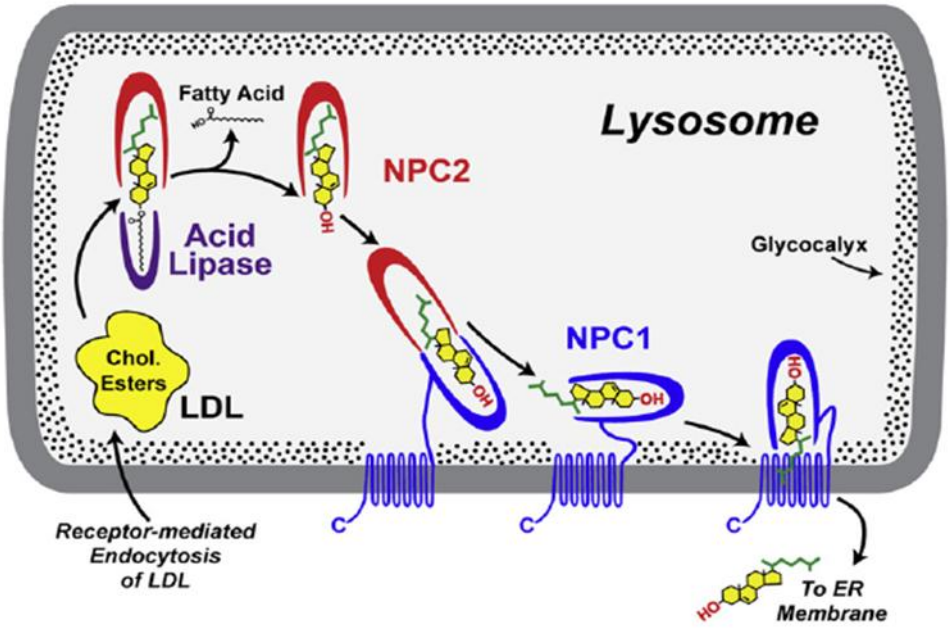


Figure 1.4- Proposed pathway for sequential transfer of cholesterol from LDL to NPC2, NPC1 and membranes. Adapted from [35].

This model is so far only a theory, because the exact functions of the NPC1 and NPC2 proteins are still unclear, which greatly complicates the understanding of the pathophysiology of this disease.

In contrast to several other lipidosis that result from defects in enzyme activity, NPC seems to represent a primary transport defect. The failure of cholesterol homeostasis in NPC cells is known but whether the cholesterol transport defect is the main problem or potentially a consequence of some other malfunction remains unclear [6].

2.4. Cholesterol transport defects in NPC

Several studies demonstrate a disruption in intracellular transport of endocytosed cholesterol in cultured skin fibroblasts from NPC patients. After endocytosis, LDL are transported to late endosomes/lysosomes, where they are hydrolysed, and free cholesterol can be released. The cholesterol in normal cells is quickly transported out of endosomes and delivered to the plasma membrane, ER and Golgi complex. In NPC disease the cholesterol is not able to exit the endocytic pathway and is accumulated within lysosomes (Figure 1.5).

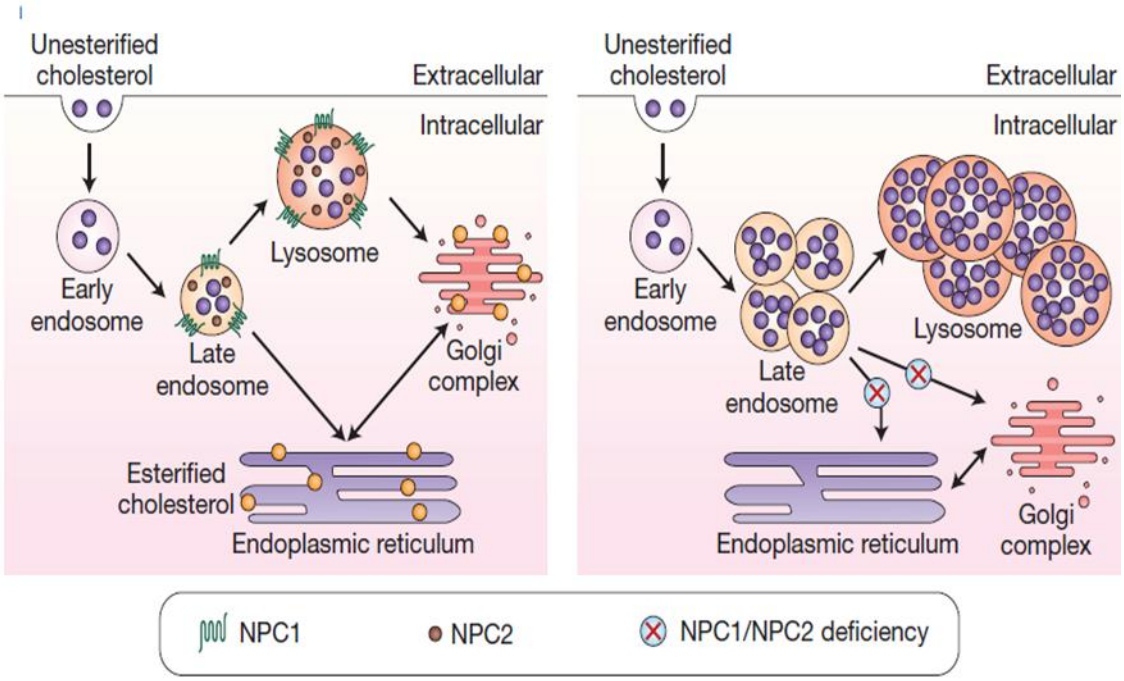


Figure 1.5 - Lipid trafficking defects in NPC. Adapted from [1].

This defect in trafficking constitutes the hallmark of the disease. Owed to this storage, there are a retarding in the LDL cholesterol-mediated homeostatic responses (more specifically cholesteryl ester formation). Normal responses can be induced by membrane

permeable oxysterol and by mevalonate, showing that the ability of the cell to respond is maintained [2].

This distinctive deficiency in processing and utilization of cholesterol by the cells plays a key role in the pathogenesis of the NPC disease. It may explain a more general dysfunction of intracellular metabolism of lipids, at least for extraneuronal organs [36]. Accumulation of cholesterol might also modulate endosome proteins such as Rab9 and mannose-6-phosphate receptors, two important proteins for the normal function of the endossomal/lysosomal system. There are some evidences that accumulation of cholesterol in late endosomes can impair vesicular trafficking pathways [37].

The pathogenesis of neuronal dysfunctions seems to be far more complex. The brain does not take up serum lipoproteins for cholesterol supply. Practically all cholesterol in the central nervous system is synthesized *de novo* [6]. Some studies demonstrated that cholesterol accumulated in cell bodies but its levels decreased in distal axons leading to an imbalance in the distribution [38]. There are also some evidences that cholesterol synthesized *de novo* may contribute significantly to the cholesterol accumulation in various cells types including glia cells [39].

2.5. Lipid storage

Although cholesterol has a major role in NPC cells anomaly, this lipid is not the only one involved in the disease. Indeed, other lipids such as sphingolipids accumulate in cells with NPC phenotype [11].

Sphingolipids are important structural and regulatory components of cell membranes. They are considered as bioactive lipids since the variation of their levels results in modifications in cellular functions [40]. In the last decades, sphingolipids have emerged as important players in different cellular processes, such as cellular growth, aging, cell death, nutrient transport, mitochondrial function, oxidative stress, angiogenesis and inflammation [41]. Sphingolipids have an amphipathic nature and are composed by a long chain sphingoid base (LCB), a fatty acid and a polar head group. The nature of LCBs and fatty acids vary between organisms. Mammalian sphingolipids differ in chain length, degree of saturation and hydroxylation whereas yeast has more strict unsaturated 26 carbons long fatty acids [42].

The major sphingolipids accumulated in NPC cells are glycosphingolipids, sphingomyelin and sphingosine [7,8,11]. The heterogeneity of storage material in NPC is well reflected in the brain, where the major storage lipids are GSL. There are many possible reasons for this event, among which are altered vesicular transport and impaired catabolism [11]. The question is if GSL contribute directly to the disease. Gangliosides (GM2 and GM3) are the more relevant GSL stored in other lysosomal storage disease LSD, being responsible for the observed severe neuropathology. To test if the gangliosides accumulation has a major role in the neuronal dysfunction of NPC disease, NPC1 mouse was crossed with mice lacking the polypeptide N-acetylgalactosaminyl-transferase (GalNac transferase), the enzyme responsible for the synthesis of GM1, GM2 and more complex gangliosides. Although the descendent mice are unable to synthesize complex gangliosides, the phenotype of the disease remain the same, suggesting that gangliosides *per se* are not responsible for the neuronal dysfunctions in this disease [43].

Sphingomyelin is elevated in peripheral tissues. Storage of SM together with the reduced activity of acid sphingomyelinase gave the name originally to the disease. There is a relationship between SM and cholesterol that is shown by the effects of adding sphingomyelinase to cellular plasma membranes leading to release of cholesterol. One hypothesis would be that SM acts as a molecular trap for cholesterol and SM storage is responsible for cholesterol elevation in NPC disease [11].

Sphingosine is a potent inhibitor of PKC and calcium channels. Sphingosine levels are increased in all tissues in NPC disease [11]. There is an increase of 12-fold in periphery and 4-fold in the brain. There is a higher possibility that sphingosine bases are involved in early stages of NPC1 pathology, since adding sphingosine to normal cells induces NPC phenotype. In fact, sphingosine is the only lipid (of all the storage lipids in NPC cells) that is capable of inducing a NPC phenotype in normal cells when added at the concentration found in NPC cells [44]. NPC1 inactivation leads to the rapid primary accumulation of sphingosine that causes a defect in lysosomal calcium storage, probably due to sphingosine mediated PKC inhibition. The lack of appropriate calcium release from the LE/Lys compartment leads to trafficking defects that in turn cause secondary storage of other lipids including cholesterol and GSL. Neurodegeneration may result from defects in all or any combination of these events (Figure 1.6).

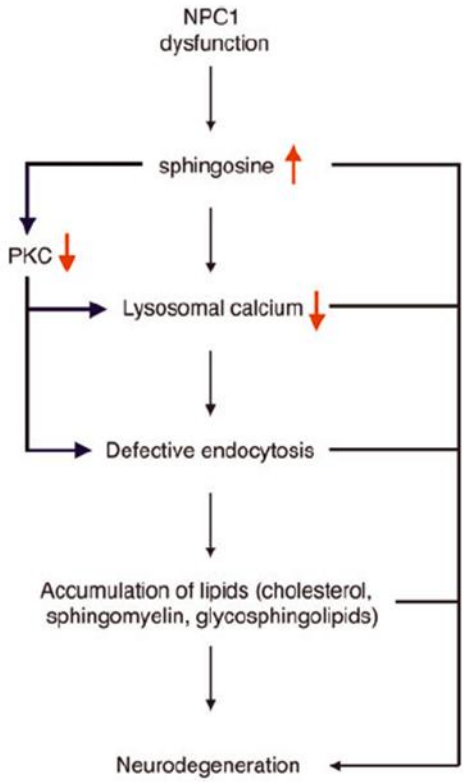


Figure 1.6 - Putative pathogenic cascade of NPC1 dysfunction. Role of the accumulation of sphingosine in the disease. Adapted from [11].

3. Oxidative stress in neurodegenerative disorders

As mentioned above, the pathogenic mechanism associated to the accumulation of intracellular lipids that leads to cell death in NPC disease is still unclear. However, there are some evidences that implicate oxidative stress in NPC [45] and other neurodegenerative disorders, such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis [46].

Oxygen (O_2) is indispensable for the majority of living organisms, as it is required for energy production in mitochondria. Molecular oxygen itself is not toxic, but its partial reduction can generate reactive oxygen species (ROS). During mitochondrial respiration, there is electron leakage from the electron transport chain that results in the generation of ROS. This process under physiologic conditions is the major intracellular source of ROS, although there are substantial controversies regarding the exact amounts of ROS produced at the different sites of the respiratory chain [47,48]. The main function of

mitochondria is the production of energy by oxidative phosphorylation. Mitochondria are responsible for producing most of the ATP necessary for cell functions. In addition mitochondria can produce and control the cellular redox state, and mantain intracellular calcium homeostasis. Oxidative stress damages mitochondrial DNA (mtDNA), and an already damaged mitochondria might incur more oxidative damage causing a vicious cycle that results in even more ROS production [49,50]. A change in the mitochondrial membrane potential is a key feature of the early stages of mitochondrial dysfunction [51]. A number of external agents can trigger ROS production, such as an increased oxygen pressure, ionizing radiation, environmental toxins (e.g., paraquat or menadione) or UV light [52].

ROS include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2). In the presence of reduced redox active transition metals, such as iron (Fe^{2+}) and copper (Cu^{+}), H_2O_2 is reduced to produce the highly reactive and powerful oxidant $OH^{\bullet}$ (Fenton reaction). The oxidised form of the metal can then be reduced by the $O_2^{\bullet-}$, via Haber-Weiss reaction:

Fenton reaction: $Metal^{n+} + H_2O_2 \rightarrow Metal^{n+1} + OH^{\bullet} + OH^{-}$

Haber-Weiss reaction: $Metal^{n+1} + O_2^{\bullet-} \rightarrow Metal^{n} + O_2$

Cellular response to oxidants depends on many factors, including cell type, the absolute level and duration of oxidant production, the specific intracellular site of ROS production, and the ROS species generated [53].

To counterbalance the generation of ROS and prevent its harmful effects, cells have developed several strategies like the production antioxidant defences. These defences include enzymes that directly detoxify ROS (e.g., superoxide dismutases, catalase, glutathione peroxidases, thioredoxin peroxidases), proteins that act as redox regulators of protein thiols (e.g., thioredoxin) and small antioxidant molecules (e.g.,glutathione). In addition, enzymes with the same biochemical antioxidant activity can be found in different cellular compartments and they are responsible for specialized detoxification of ROS at specific compartments [54]. Under physiological conditions, a balance is established between ROS levels and cellular antioxidant defences. This allows the cell to restrains the pro-oxidant effects of ROS. But, if the ROS production is increased, or the antioxidant defences are reduced, cells face an imbalance of the homeostasis in favour of ROS. This will lead an oxidative stress and the concomitant accumulation of oxidative damages to lipids, DNA and proteins [54,55].

The CNS is particularly sensitive to oxidative stress. This can be explained by several features of the CNS: the susceptibility to lipid peroxidation, because of the higher concentration of unsaturated fatty acids, the fact that neurons require large amounts of energy that leads to high oxygen consumption and ultimately production of ROS, and the lower levels of antioxidant defences, such as glutathione, compared with other organs [56,57]. Mitochondrial dysfunction leading to oxidative stress appears to be a major factor in many neurodegenerative diseases, such as Alzheimer disease [58] and Parkinson Disease [59], and pathologies associated with liver and cardiac damage [45].

3.1. Oxidative stress in NPC

Oxidative stress had been implicated in NPC. It was shown an imbalance in the intracellular redox state, both in fibroblasts from NPC patients and in experimentally-induced NPC1 knockdown in vitro (using small interference RNA - siRNA) in primary cultures of fibroblasts [60]. An increase in ROS concentration was also found in human SHSY5Y neuroblastoma cells, induced with the same NPC1 knockdown, suggesting that probably the imbalance in the intracellular redox state would not be restricted to a single cell type [60]. There are also evidences of an increase in oxidative stress in vivo in the cerebellum of $NPC1^{-/-}$ mice [61].

Mitochondrial dysfunction seems to have an important role in NPC disease. Fu and collaborators have shown that Coenzyme Q10 may contribute directly to NPC pathology. In the mitochondrial respiratory chain, CoQ10 plays an important role in the production of chemical energy, and an increase in ubiquinone-10 (oxidized) and the respective decrease in ubiquinol-10 (reduced) in NPC cells suggests mitochondrial dysfunction [62]. One study showed a decrease in catalase activity in several NPC models, leading to an increase of intracellular levels of ROS and lipid peroxidation [60]. Notably, antioxidant therapies failed to attenuate oxidative stress in NPC cells or animals [63,64].

Another study suggests that neuronal dysfunctions and degeneration in $NPC1^{-/-}$ mouse brains are caused by a decrease in mitochondrial membrane potential that leads to a reduction in ATP synthesis. In addition, mitochondrial dysfunctions have been associated to increased levels of cholesterol in the membrane of the mitochondria. When the levels of mitochondrial membrane cholesterol were decreased to the levels in $NPC1^{+/+}$ cells, ATP synthesis and mitochondrial function were restored, supporting the hypothesis that increased mitochondrial cholesterol may cause dysfunctions *per se* [65].

4. Yeast as a biological model for NPC

The yeast *Saccharomyces cerevisiae* is a unicellular eukaryote fungus used as experimental model organism in scientific research. It has been used by men for a very long time in the production of economic valuable products. The similarities in organelles and biomolecules with higher eukaryotes makes yeast a useful model for the study of processes conserved throughout evolution [54]. *S. cerevisiae* has been used as a model to study biological processes such as cell cycle control [66], stress responses [55], or even mechanisms underlying neurodegenerative diseases [67].

The first organism to have its genome fully sequenced was *S. cerevisiae*. The availability of the several databases that contain information about this microorganism genes and proteins, such as Saccharomyces Genome Database, provides a wide range of knowledge about protein-protein interaction, genetic interactions, protein functions and predict orthologues in other organisms [68]. The development of genomic and proteomic tools makes very easy to manipulate yeast genetically, and techniques for the manipulation are strongly optimized [69].

The yeast *Saccharomyces cerevisiae* has a single copy of *NCR1*, a NPC1 orthologue. Ncr1p contains multiple transmembrane domains, such as NPC1 domain, a conserved SSD domain [70], and a cysteine-rich domain [71] (Figure 1.7). Moreover, NPC1 proteins have an extremely high functional conservation among species. Indeed, Ncr1p is able to suppress cholesterol and ganglioside accumulation when expressed in NPC1-deficient Chinese hamster ovary cells [70]. Most of the NPC1 mutations occur in amino acids that are conserved in yeast Ncr1p. Indeed, Ncr1p presents a rate of identity and similarity of 34% and 57%, respectively, when compared with human NPC1 [23,72]. Furthermore, of 105 identified miscoding patient mutations, 66% of the affected amino acids are conserved in yeast and of these, 50% are identical between Ncr1p and human NPC1 [71].

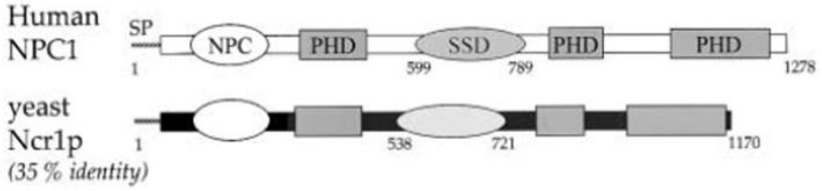


Figure 1.7 – Comparisons of the NPC1 and Ncr1p protein sequences [70].

Since this thesis will focus on the role of sphingolipid signalling in mitochondrial function and oxidative stress resistance in a yeast model of NPC, an overview of this subject will be provided in the next section.

4.1. Sphingolipid signalling and mitochondria function in yeast

In yeast the sphingolipid metabolism (Figure 1.7) has an unique entry point, catalysed by the serine palmitoyltransferase (SPT), and one exit point, catalysed by the sphingoid base phosphate lyase, which generates non sphingoid molecules [40]. The first reaction in sphingolipid biosynthesis occurs in the endoplasmic reticulum and consists in the condensation of serine and palmitoyl-CoA, catalysed by SPT, generating 3-ketodihydrosphingosine (3-keto-DHS), CO2 and coenzyme A (CoA). Readily after its formation, 3-keto-DHS is reduced in the ER to DHS by a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent 3-ketodihydrosphingosine reductase. DHS can produce phytoceramide by two different routes. It can be hydroxylated by Sur2p/Syr2p hydroxylase in order to form the other LCB, PHS, and then acylated to form phytoceramide by ceramide synthase. Alternatively, DHS can be acylated by ceramide synthase to form dihydroceramide prior to its hydroxylation into phytoceramide. Ceramides must be transported from the ER to the Golgi apparatus in order to receive their polar heads to form complex sphingolipids. The mechanism of transport in yeast is still unclear [42]. Yeast and mammalian complex sphingolipids differ in their polar heads. Yeast synthesizes inositophosphosphingolipids that may contain mannose whereas mammalian cells produce sphingomyelin through the transfer of choline phosphate to ceramide. The mammalian cells ceramide can be converted to glucosylceramide to yield later complex glycosphingolipids) or it can also be phosphorylated to ceramide-1-phosphate [42,73].

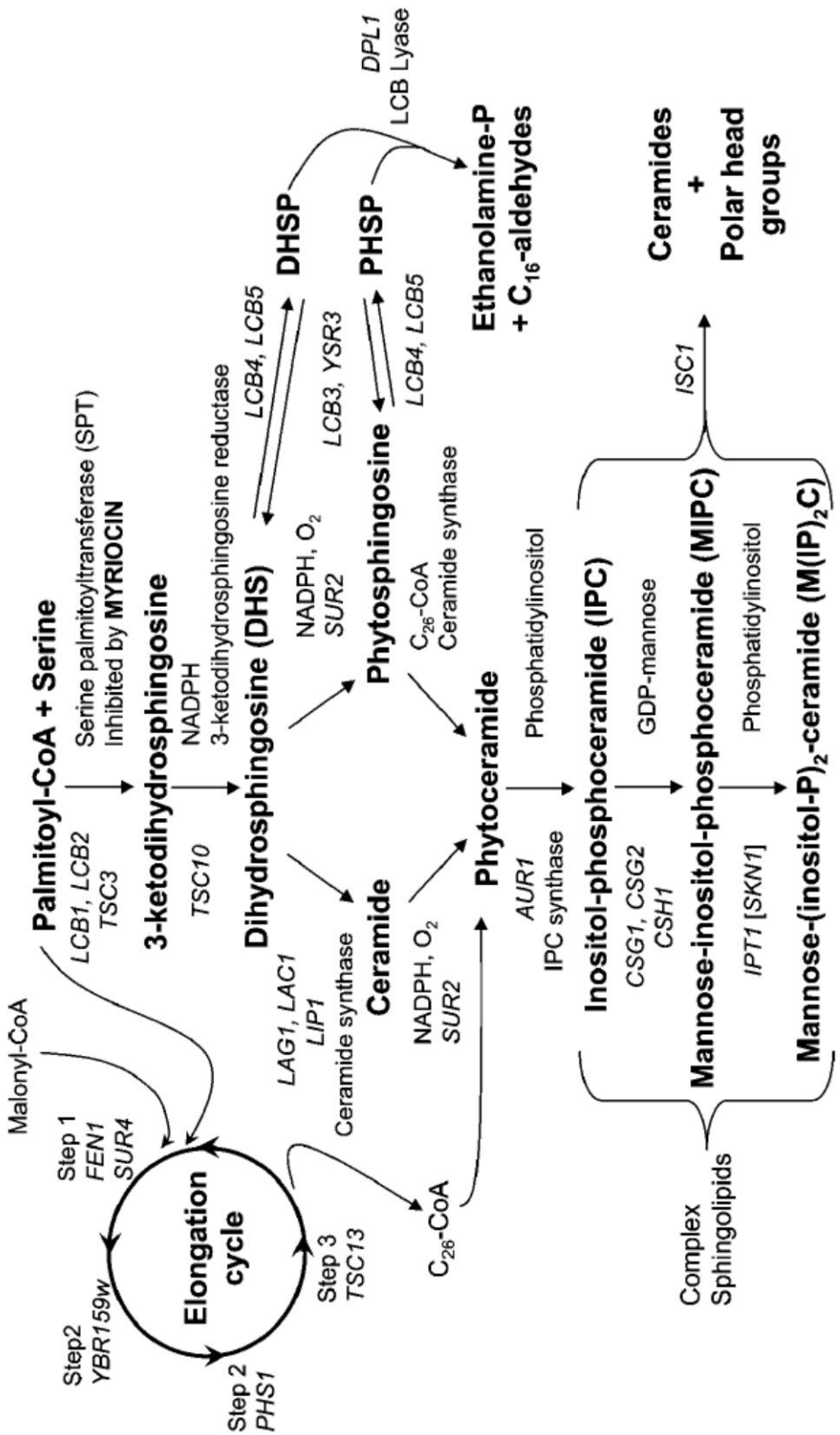


Figure 1.8 - Sphingolipid synthesis in *Saccharomyces cerevisiae* [73].

Although the regulation of mitochondrial function is very complex, numerous studies suggest that signal transduction pathways modulated by nutrients and sphingolipids play a key role in this process (Figure 1.9). Components of these signalling pathways include the PIK-related Tor1p protein kinase subunit of the rapamycin-sensitive TORC1 complex [74], the catalytic subunit of the ceramide-activated protein phosphatase Sit4p [75], and the Sch9p kinase, a homolog of mammalian ribosomal S6 kinase and mammalian PKB/Akt [76].

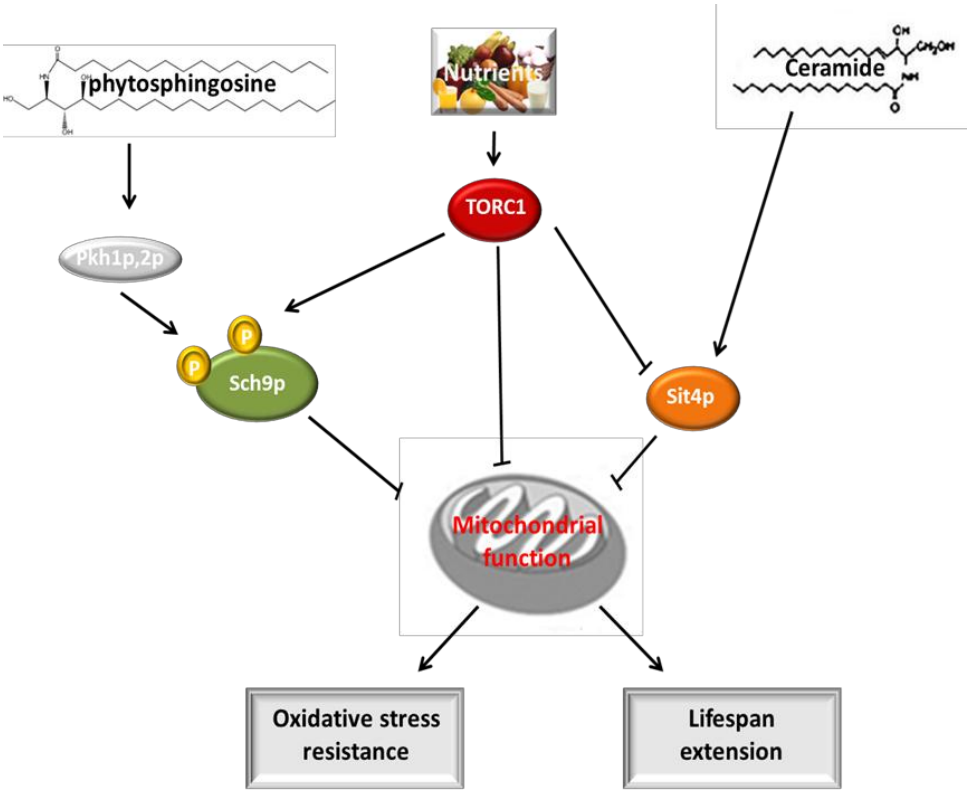


Figure 1.9 - Crosstalk between nutrient and sphingolipid signalling pathways that control mitochondrial function. Tor1p resides in a multi-protein complex TORC1 and is activated by nutrients, in particular by amino acids. This protein by phosphorylation activates Sch9p and inactivates Sit4p. Sch9p and Sit4p are also regulated by sphingolipids, namely ceramide and phytosphingosine respectively. All proteins inactivate mitochondrial function, decreasing oxidative stress resistance and lifespan.

4.1.1. Tor1p

The Target of Rapamycin (TOR) is a serine-threonine protein kinase conserved in all the eukaryotes. Studies in yeast revealed that TOR resides in two distinct multi-protein complexes, TORC1 (sensitive to rapamycin), and TORC2 (insensitive to rapamycin).

These complexes function in separate signalling pathways and regulate distinct aspects of the cell growth. Together these pathways define the TOR signalling network [77].

The activity of TORC1 is influenced by growth factors, energy and cellular stress. It positively controls proteins synthesis regulating ribosome biogenesis at multiple levels, translation of some genes, and metabolism (amino acids biosynthesis and glucose homeostasis) [78]. TORC1 activity is influenced by nutrients, mainly by the nitrogen source. The main suggestion for this connection is that treatment of cells with rapamycin has the same effects of nitrogen deprivation on transcription, development and metabolism. For instance both nitrogen deprivation and rapamycin treatment induce autophagy, exit from the cell cycle and growth arrest at G0, and changes in transcription of some genes [79]. Despite uncertainty in the position of TORC1 within the nutrient signalling pathway, TORC1 certainly plays a major role in transition between growth and quiescence.

TORC1 can regulate directly important proteins such as Sit4p and Sch9p. These proteins have a role in oxidative stress resistance, chronological lifespan (CLS) and mitochondrial function [80].There are complex relationships between TOR signalling, mitochondrial function, ROS production, and yeast CLS. In fact, the deletion of *TOR1* increases CLS as well oxidative stress resistance [74,81,82]. The absence of Tor1p forces the yeast to make respiration instead of fermentation, increasing mitochondrial activity and decreasing the production of ROS. In contrast, the activation of this pathway induces an increase in ROS production, damaging the mitochondria, which in turn results in a higher production of ROS [83].

4.1.2. Sit4p

In yeast, Sit4p is the catalytic subunit of the ceramide-activated protein phosphatase, a heterotrimeric complex that also includes Tpd3p and Cdc55p as regulatory subunits [84]. Sit4p is a serine-threonine protein phosphatase related to type 2A family of protein phosphatases (PP2A), and it has a high homology to other protein phosphatases, including the fission yeast PP2A and human protein phosphatase 6 that are involved in cell cycle regulation. Sit4p has a role in the control of the Swi4p factor that regulates the transcription of G1 cyclin genes and is required for down regulation of cell functions that depend on Pkc1p, such as cell wall integrity pathway, cytoskeleton organization, and ribosomal gene expression [85,86].

Yeast cells lacking the Sit4p are unable to grow under anaerobic conditions, indicating that the energy produced by mitochondrial respiration is essential for their viability [87]. A recent study showed that loss of Sit4p suppresses the shortened CLS,

oxidative stress sensitivity and mitochondrial dysfunctions of cells lacking Isc1p protein, an orthologue of the mammalian neutral sphingomyelinase 2 [75].

4.1.3. Sch9p

Sch9p is a serine-threonine kinase with possible homology to mammalian ribosomal S6 kinase (S6K) [88] or Protein Kinase B (PKB/AKT) [89]. The activation of Sch9p induces ribosome biogenesis genes and inhibits genes involved in carboxylic acid metabolism. Glucose affects Sch9p function, both by increasing the level of Sch9p in the cell and by inducing its phosphorylation Sch9p phosphorylation as also induced by stress conditions such as heat stress [73,90]. Sch9p protein can also be activated by sphingolipids such as phytosphingosine through Pkh1/2p pathway [73,80]. Nutrients, more specifically amino acids, can activate Sch9p through TORC1 pathway, promoting antagonistic effects when compared with rapamycin and starvation treatments (two known inhibitors of TORC1), which rapid lead to a dephosphorylation of Sch9p. In addition recent studies showed that TORC1 directly phosphorylates Sch9p at multiple sites in its C-terminal domain [88].

Deletion of *SCH9* gene leads to phenotypes similar to the observed in cells lacking Tor1p,improving mitochondrial function, decreasing ROS levels, extending CLS and increasing resistance to acid, thermal and oxidative stress conditions [74,76,88,91].

Chapter 2

Scope of the work

Scope of the Work

Previous studies implicate mitochondrial dysfunction and oxidative stress in NPC disease [60–62,65]. Moreover, changes in the level of sphingolipids have been described in NPC models [11].

Thereby, the present work aimed to:

i. uncover the role of the yeast Ncr1p in oxidative stress resistance
ii. characterize changes in mitochondrial function in Δ*ncr1* cells
iii. characterize the role of Tor1p, Sit4p and Sch9p in the dysregulation of mitochondrial function and oxidative stress resistance in Δ*ncr1* cells

Chapter 3

Material and Methods

1. Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 3.1. Yeast cells were grown aerobically at 26 ºC in a gyratory shaker (at 140 rpm), with a ratio of flask volume / medium volume of 5:1, to early exponential phase (OD_{600nm}=0.6). The growth media used were: yeast peptone dextrose, YPD, (1 % (wt/vol) yeast extract, 2 % (wt/vol) bactopeptone, 2 % (wt/vol) glucose); synthetic complete (SC) drop-out medium containing 2 % (wt/vol) glucose and 0.67 % yeast nitrogen base without amino acids and supplemented with appropriate amino acids or nucleotides (0.008 % (wt/vol) histidine, 0.008 % (wt/vol) tryptophane, 0.04 % (wt/vol) leucine and 0.008 % (wt/vol) uracil) and minimal medium (0.67 % (wt/vol) yeast nitrogen base without aminoacids, 2 % (wt/vol) glucose), supplemented with appropriate amino acids and nucleotides (0.004 % (wt/vol) histidine, 0.004 % (wt/vol) methionine, 0.008 % leucine and 0.004 % (wt/vol) uracil)].

For the spotting assay, cells were diluted to OD_{600}nm=0.1 and fivefold serial dilutions were plated on supplemented with SC - 2 % (wt/vol) glucose (control) or SC - 3 % (wt/vol) glycerol.

Table 3.1 – Yeast strains used in this work

Strain	Genotype	Reference/source
BY4741	Mata, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	EUROSCARF
Δ*ncr1*	BY4741Δ*ncr1*::KanMX4	Vilaça, R
Δ*ncr1*	BY4741Δ*ncr1*::URA3	This study
BY4741 pRS316	BY4741 carrying pRS316	Vilaça, R.
BY4741 pNCR1	BY4741 carrying pRS316-NCR1	Vilaça, R.
Δ*ncr1* pRS316	Δ*ncr1* carrying pRS316	Vilaça, R.
Δ*ncr1* pNCR1	Δ*ncr1* carrying pRS316-NCR1	Vilaça, R.
Δ*tor1*	BY4741 Δ*tor1*::*KanMX4*	EUROSCARF
Δ*ncr1*Δ*tor1*	BY4741Δ*ncr1*::*URA3* Δ*tor1*::*KanMX4*	This study
Δ*sit4*	BY4741 Δ*sit4*::*KanMX4*	EUROSCARF
Δ*ncr1*Δ*sit4*	BY4741Δ*ncr1*::*URA3* Δ*sit4*::*KanMX4*	This study
Δ*sch9*	BY4741 Δ*sch9*::*KanMX4*	EUROSCARF
Δ*ncr1*Δ*sch9*	BY4741Δ*ncr1*::*URA3* Δ*sch9*::*KanMX4*	This Study
W303a	Mata, *ura3*-1, *leu2*-3, *his3*-11, *trp*1-1, *ade2*-1, *can1*-100	[92]
W303aΔ*ncr1*	W303a Δ*ncr1*::*KanMX4*	This study

2. Oxidative stress resistance

Yeast cells were grown to exponential phase (OD_{600nm}=0.6) and treated with H_2O_2 for 1 h. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5 % agar. Colonies were counted after growth at 26 ºC for 3 days. Viability was expressed as the percentage of the colony-forming units (CFU).

3. Genomic DNA extraction

Cells (10 mL) were grown to stationary phase and harvested by centrifugation during 5 min at 4000 rpm. The pellet was collected, washed once and resuspended in 100 µL of lysis buffer (2 % (vol/vol) Triton X-100, 1 % (wt/vol) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 100 µL of phenol:chloroform:isoamyl alcohol (50:48:2). Cells were lysed by vigorous shacking of the cell suspension in the presence of glass beads for 3 min (short pulses of 1 min were used, with 1 min intervals). The aqueous phase was recovered after centrifugation at 4000 rpm 5 min, and 100 µL of chloroform were added. The mixture was homogenized by vortexing 3 min (as described previously), supplemented with 100 µL TE 10x (100 mM Tris, 10 mM EDTA pH 8.0) and centrifuged (14000 rpm, 5 min). The aqueous phase was washed with 1 mL of 100 % ethanol. After centrifugation (14000 rpm, 3 min), the pellet was resuspended in 400 µL of TE 1 x. It was added 30 µg of RNAase and the mixture was incubated 5 min at 37 ºC. Then, 10 µL of 4 M ammonium acetate and 1 mL of 100 % ethanol were added. The DNA was collected by centrifugation (14000 rpm, 3 min), washed twice with 70 % (vol/vol) ethanol, dried and resuspended in appropriate water. The genomic DNA was quantified using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific) and analyzed by gel electrophoresis in 1.4 % (wt/vol) agarose.

4. PCR

A mix of 20 µL containing 1 x Reaction Buffer (Thermo Scientific), 1.5 mM $MgCl_2$ (Thermo Scientific), 0.2 mM sense primer, 0.2 mM antisense primer, 0.2 µM dNTPs (Fermentas), 1 U Taq Polymerase (Thermo Scientific), and 150 ng genomic DNA was prepared. All the primers used in this study except *TOR1*_amp_Fw and *TOR1*_amp_Rv have an annealing temperature of 55 ºC and an elongation of 2 min during 30 cycles. For the amplification of *KanMX4* the annealing temperature was 58ºC, the elongation was 2min20s, during 35 cycles. PCR products were analysed in agarose gel 0,8 % (wt/vol) using TAE 1 x as buffer, and DNA bands were compared to Gene Ruler Ladder Mix (Fermentas).

5. Gene disruption

The disruption of *NCR1* using *CaURA3* cassette was performed by homologue recombination in Δ*ncr1* (with *KanMX4*). The deletion fragment containing *URA3* and flanking regions of *KanMX4* was amplified, from the plasmid pAG61 containing the *C. albicans URA3* ORF [93], by PCR using the primers *CaURA3*_amp_Fw and *CaURA3*_amp_Rv. The disruption of *TOR1* gene in Δ*ncr1* cells was performed by homologous recombination, using a deletion fragment containing *KanMX4* and the flanking regions of *TOR1*. This deletion fragment was amplified by PCR using the primers *TOR*1_amp_Fw and *TOR1*_amp_Rv (Table 3.2) and genomic DNA from Δ*tor1* cells. For *NCR1* gene disruption in Δ*sit4* and Δ*sch9* cells, a deletion fragment containing *URA3* and the flanking regions of *NCR1* was amplified by PCR using primers *NCR1*_amp_Fw and *NCR1*_amp_Rv (Table 3.2) and genomic DNA from Δ*ncr1* cells (with URA3). Purification of DNA from TAE agarose gel was performed with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Cells were transformed by electroporation (as described below) and mutant cells were selected in rich medium (YPD) containing geneticin (*TOR1* deletion), or minimal medium lacking uracil (*NCR1* deletion). Gene disruption was confirmed by PCR, using the fallowing pars of primers: *TOR1*_conf_Fw + Int*KanMX4*_conf_Rv or *TOR1*_conf_Fw + Int*TOR1*_conf_Rv (for *TOR1* deletion); *NCR1*_conf_Fw + Int*URA3*_conf_Rv or *NCR1*_conf_Fw + Int*NCR1*_conf_Rv (for *NCR1* deletion) (Table 3.2).

Table 3.2 – Primers used in this work

Primers	Sequence
***CaURA3*_amp_Fw**	TCCTTGACAGTCTTGACG
***CaURA3*_amp_Rv**	GTATAGCGACCAGCATTC
***TOR1*_amp_Fw**	GAGAATCATTACCGGCGAAA
***TOR1*_amp_Rv**	TACGAACACGTTTGGTGATG
***NCR1*_amp_Fw**	CCGTGGCTAATGTCACAACA
***NCR1*_amp_Rv**	TTACGAGTGAAGCGTTCTGG
***TOR1*_conf_Fw**	CAAAGAGTAAGAGGGTCCTTGGG
Int*TOR1*_conf_Rv	GAACAGCCCCTACCCTAGTGCT
Int*KanMX4*_conf_Rv	TGCTGTTTTGCCGGGGAT
***NCR1*_conf_Fw**	AAGGTGCGAAATGACGGAAGA
Int*NCR1*_conf_Rv	CGTCGTCCACAATCATTGCCC
Int*URA3*_conf_Rv	CCCAGTGACACCATGAGCATTAG

*Fw - Forward /Rv - Reverse

6. Yeast electroporation

6.1. Preparation of electrocompetent cells

Cells were grown in 50 mL of YPD medium to an OD_{600nm} = 1.3 – 1.5, harvested, and resuspended in 10 mL of 10 mM Tris 1 mM EDTA 100 mM lithium acetate pH 7.5 and gently shacked during 45 min at 30 ºC. Then, 250 µL of 1 M DTT was added and cells were shacked 15 min at 30 ºC. Ice-cold sterile water was added for a final volume of 50 mL and cells were centrifuged at 4 ºC. Cells were washed first with 25 mL of ice-cold sterile water and then with 2 mL of 1 M sorbitol (4 ºC), and resuspended in 50 µL of 1 M sorbitol (4 ºC).

6.2. Electro-transformation and plating

Electrocompetent cells (40 µL) were mixed with 5 µL of deletion fragment (± 0.5 µg) and incubated on ice for 5 minutes. The mixture was transferred to prechilled sterile 2 mm electroporation cuvette. An electric pulse (1.5 kV, 25 µF and 200 Ω) was applied in parallel using an electroporation system (BioRad). After the pulse delivery, 1 mL of selective minimal medium containing 1 M sorbitol was immediately added and cells were gently shacked for 4 h at 26 ºC. Cells were plated in selective medium and grown at 26 ºC for 3 days.

7. Oxidative stress markers

7.1. Quantification of ROS

This method is based on an in vivo intracellular oxidation of the oxidant-sensitive probe dihydroethidium (DHE) (Molecular Probe, Life Technologies).

Yeast cells were grown to exponential phase, exposed to 1.5 mM of H_2O_2 for 50 minutes at 26 ºC and labelled with 1 µL of 5 mM DHE for 10 minutes at 26ºC. Cells were washed twice with filtered PBS and sonicated. Fluorescence was measured on the FL-3 channel of a Becton-Dickinson FACSort flow cytometer using 15.000 events. The data was analyzed using the FlowJo software (Tree Star) and the percentage of positive cells was quantified, using as control the autofluorescence of the cells.

7.2. Lipid peroxidation

Lipid peroxidation was determined by quantifying thiobarbituric acid reactive substances as described [94]. This method is based on the acid-catalyzed decomposition of lipid peroxides to malondialdehyde (MDA) which reacts with thiobarbituric acid to form a red chromogen.

Yeast cells were harvested by centrifugation and yeast extracts were prepared in 50 mM potassium phosphate buffer (pH 7.0), by vigorous shaking of the cell suspension in the presence of glass beads for 3 min. TCA were added and the shaking were performed for 2 min. Short pulses of 1 min were used, with 1 min intervals on ice. Briefly, the following mixture was made for each sample: 100 µL sample or buffer (control) + 100 µL 0.1 M EDTA + 600 µL thiobarbituric acid solution (26 mM TBA, 0.25 M HCl, 15 % TCA) + 8.1 µL 50 mM BHT. The mixtures were boiled for 15 min and chilled out at ambient temperature. Absorbance was measured at 532 nm [95]. The MDA concentration was estimated using $\varepsilon = 1{,}56x105\ M^{-1}\ cm^{-1}$.

7.3. Carbonylation

Protein oxidation was determined by immunodetection of protein carbonyls, as previously described [96].This method is based on the reaction of carbonyl groups with 2,4 - dinitrophenylhydrazine (DNPH) to form 2,4 – dinitrophenylhydrazone (DNP).

Yeast cells were harvested by centrifugation and yeast extracts were prepared in 50 mM potassium phosphate buffer (pH 7.0) containing protease inhibitors (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boehringer Mannhein), by vigorous shaking of the cell suspension in the presence of glass beads for 5 min. Short pulses of 1 min were used, with 1 min intervals on ice. Cell debris was removed by centrifugation at

13000 rpm for 15 min and protein content was determined by the method of Lowry [97], using bovine serum albumin as a standard.

A volume corresponding to 40 µg of protein was mixed with 1 volume of 12 % (wt/vol) SDS. After a short spin, 2 volumes of 20 mM DNPH 10 % (vol/vol) TFA were added and mixed. The mixture was incubated for 30 min at room temperature and in the dark. The samples were neutralized with 1.5 volumes of 2 M Tris 30 % (vol/vol) glycerol 19 % (vol/vol) β-mercaptoethanol.

Proteins derivatized with DNPH (0.1 µg) were slot blotted onto a PVDF membrane (Hybond P^{TM}, GE Healthcare) previously activated with 100 % methanol. Alternatively, 12 µg of derivatized proteins were separated by electrophoresis using 10 % polyacrylamide gel, LMW Calibration Kit for SDS Electrophoresis (GE Healthcare) was used as protein standards. Proteins were transferred to a nitrocellulose membrane (Hybond-ECL^{TM}, GE Healthcare) previously hydrated with transfer buffer (39 mM glycine, 48 mM Tris, 0.0375 % (wt/vol) SDS, 20 % (vol/vol) methanol). Blotting was performed at 0.8 mA/cm^2 for 1 h. After blotting, the nitrocellulose membranes were stained with Ponceau S (0,6 % (wt/vol) of Ponceau S, 3 % (wt/vol) TCA and 3 % (wt/vol) sulfosalicylic acid acid) to visualize proteins. Slot blot and western blot membranes were blocked for 1 h with 5 % (wt/vol) milk powder in TPBS (0.065 % (wt/vol) Tween-20, 0.4 % (wt/vol) NaCl, 0.01 % (wt/vol) KCl, 0.09 % (wt/vol) $Na_2HPO_4.2H_2O$, 0.012 % (wt/vol) KH_2PO_4) and incubated overnight with the primary antibody rabbit IgG anti-DNP (1:1500; Sigma). Membranes were washed twice with TPBS and incubated for 1 h with the secondary antibody goat anti-rabbit IgG-peroxidase (1:5000; Sigma). Membranes were washed twice with TPBS and with TBS (0.4 % (wt/vol) NaCl, 0.01 % (wt/vol) KCl, 0.09 % (wt/vol) $Na_2HPO_4.2H_2O$, 0.012 % (wt/vol) KH_2PO_4). Immunodetection were performed by chemiluminescence, using ECL^{TM} Western Blotting Detection Reagents (GE Healthcare). Membranes were exposed to a Hybond-ECL film (GE Healthcare), and the film was developed.

8. Statistical analyses

Data were analysed in GraphPad Prism Software v5.01 (GraphPad Software) and expressed as mean values ± SD from at least three independent experiments. Values were compared by Student's t-test $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

Chapter 4

Results

1. Ncr1p deficiency decreases resistance to hydrogen peroxide

To analyse the resistance of cells lacking Ncr1p to oxidative stress conditions, *S. cerevisiae* BY4741 (parental) and Δ*ncr1* mutant cells were grown to exponential phase (OD_{600nm}=0.6) and treated for 1 hour with 0.5 or 1.5 mM H_2O_2. This oxidant agent is widely used to induce an acute oxidative stress.

As expected, the results showed a dose-dependent decrease in the viability of the parental cells (Figure 4.1 A). The deletion of *NCR1* significantly decreased the resistance of yeast cells to H_2O_2 for both concentrations.

We also evaluated H_2O_2 resistance of cells lacking Ncr1p in another background strain (W303a). The results obtained were similar to those described for the BY4741 strain, suggesting that the oxidative stress sensitivity of Δ*ncr1* mutant cells is not strain related (Figure 4.1 B).

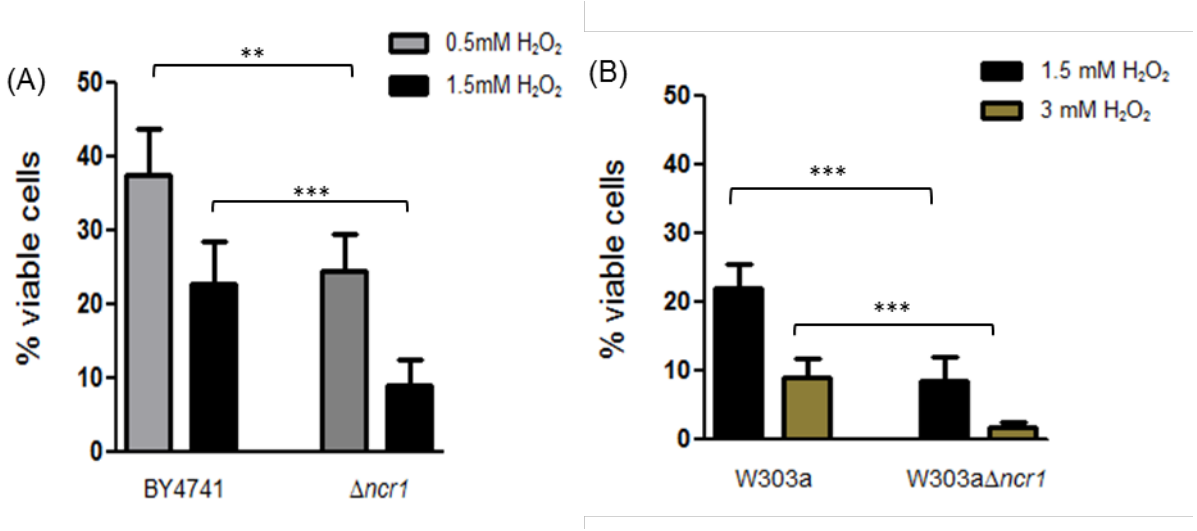


Figure 4.1- Effect of *NCR1* deletion in oxidative stress resistance. *S. cerevisiae* BY4741 **(A)** or W303 **(B)** strains and its isogenic Δ*ncr1* mutant cells were grown to exponential phase in SC-glucose medium and exposed H_2O_2 for 1 h. Cell viability was expressed as the percentage of the colony-forming units (treated cells vs. untreated cells). Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s *t-test*. **,p< 0.01; ***, p< 0.001

To evaluate if the higher sensitivity of the Δ*ncr1* mutant cells to hydrogen peroxide was specifically related with the absence of Ncr1p, oxidative stress was assessed in BY4741 and Δ*ncr1* mutant cells transformed with pRS316 (empty vector) or pRS316-*NCR1* (plasmid expressing Ncr1p under its own promoter).

The results show that *NCR1* expression in parental cells did not affect the resistance to H_2O_2 (Figure 4.2). However, the viability of Δ*ncr1* mutant cells expressing *NCR1* and treated with 0.5 or 1.5 mM H_2O_2 was significantly higher (37 % and 14 %, respectively) to the observed in Δ*ncr1*-pRS316 cells (23 % and 5 %, respectively). These results show that *NCR1* expression partially suppresses H_2O_2 sensitivity of Δ*ncr1* mutant cells, confirming the hypothesis that Ncr1p is important to H_2O_2 resistance.

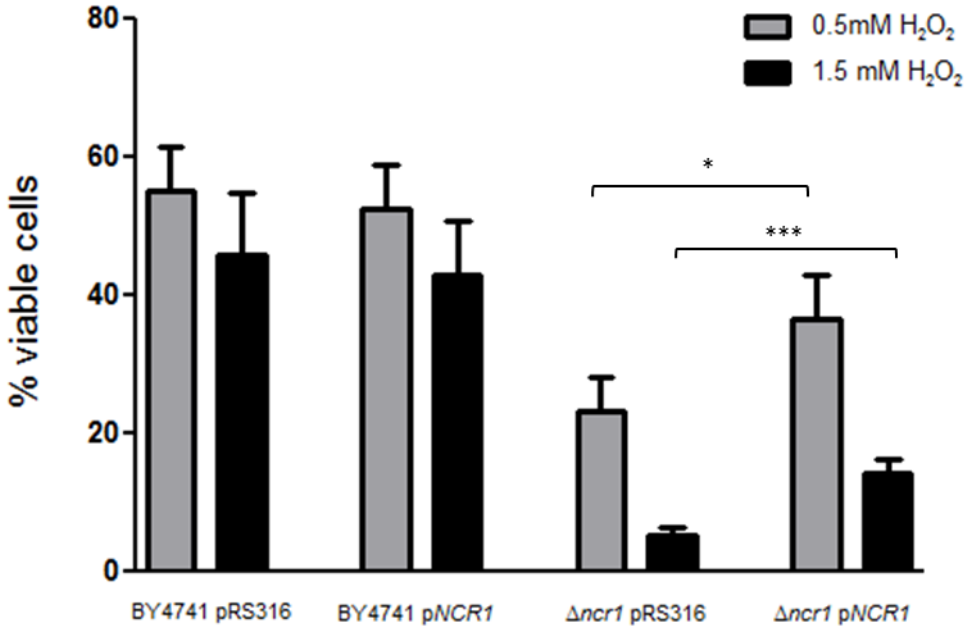


Figure 4.2- Effect of *NCR1* expression in oxidative stress resistance. *S. cerevisiae* BY4741 and Δ*ncr1* cells transformed with pRS316 or pRS316-*NCR1* were grown to exponential phase in SC-glucose medium lacking uracil and exposed to 0.5 and 1.5 mM H_2O_2 for 1 h. Cell viability was expressed as the percentage of the colony-forming units (treated cells vs. untreated cells). Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s *t-test*.*, $p< 0.05$; ***, $p< 0.001$

2. Oxidative stress markers

Hydrogen peroxide-induced cell death is associated with an increase in the levels of oxidative stress markers [96]. Therefore, we investigated if the sensitivity of Δ*ncr1* mutant cells to H_2O_2 was associated with higher levels of oxidative stress markers, namely reactive oxygen species, lipid peroxidation and protein carbonylation.

2.1. Intracellular oxidation

The intracellular levels of ROS were measured using dihydroethidium (DHE), a non-fluorescent probe that is highly sensitive to superoxide radicals. The reaction of DHE with superoxide generates ethidium [98], a fluorescent dye that can be detected by flow cytometry or spectrofluorimetry.

To test if the lower resistance of the cells lacking Ncr1p to H_2O_2 was associated with higher levels of intracellular ROS, both parental cells and Δ*ncr1* mutant cells were maturated or treated with 1.5 mM of H_2O_2, were labelled with DHE. Unlabelled cells were used as control (autofluorescence).

The quantification of DHE positive cells by fluorescence-activated cell sorting (FACS) analysis showed an increase with hydrogen peroxide treatment (Figure 4.3). However, this increase was significantly higher in Δ*ncr1* mutant cells. Moreover, the basal levels of ROS were higher in Δ*ncr1* mutant cells, compared with parental cells (Figure 4.3). These results suggest that the sensitivity of Δ*ncr1* mutant cells to H_2O_2 is associated with higher levels of intracellular ROS.

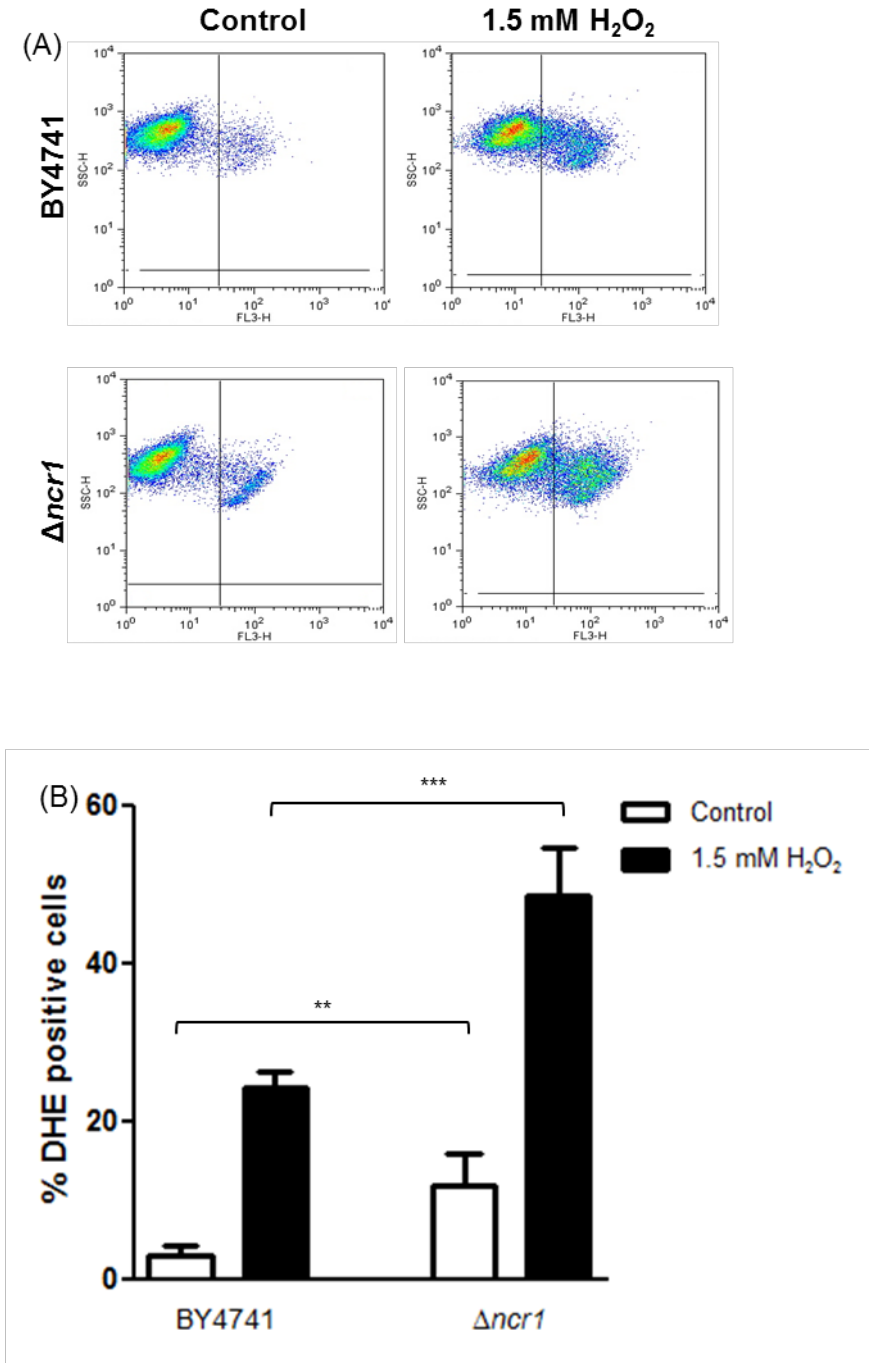


Figure 4.3 – Quantification of intracellular ROS. *S. cerevisiae* BY4741 and Δ*ncr1* cells were grown to exponential phase, treated with 1.5 mM of H_2O_2 for 50 minutes labelled with DHE for 10 minutes and analysed by FACS as described in methods. **(A) –** FACS plot analysis of cells untreated and treated with H_2O_2 **(B) –** Quantification of the intracellular ROS levels using information from FACS plots. The % of positive cells was quantified by Flowjo software analyses. Data are mean ± SD of at least three independent experiments. Values were compared by Student´s t-test. **, $p< 0.01$; ***, $p< 0.001$

2.2. Lipid peroxidation

Lipid peroxidation was assessed in BY4741 and Δ*ncr1* mutant cells untreated or treated for 1 hour with 1.5 mM H_2O_2. In parental cells, exposure to H_2O_2 increased 43 % lipid peroxidation (Figure 4.4). This increase was significantly higher (95 %) in Δ*ncr1* mutant cells. In addition, the basal levels of lipid peroxides in the mutant cells were 64 % higher to the observed in parental cells (Figure 4.4). These results suggest that the sensitivity of Δ*ncr1* mutant cells to H_2O_2 is associated with higher levels of lipid peroxidation.

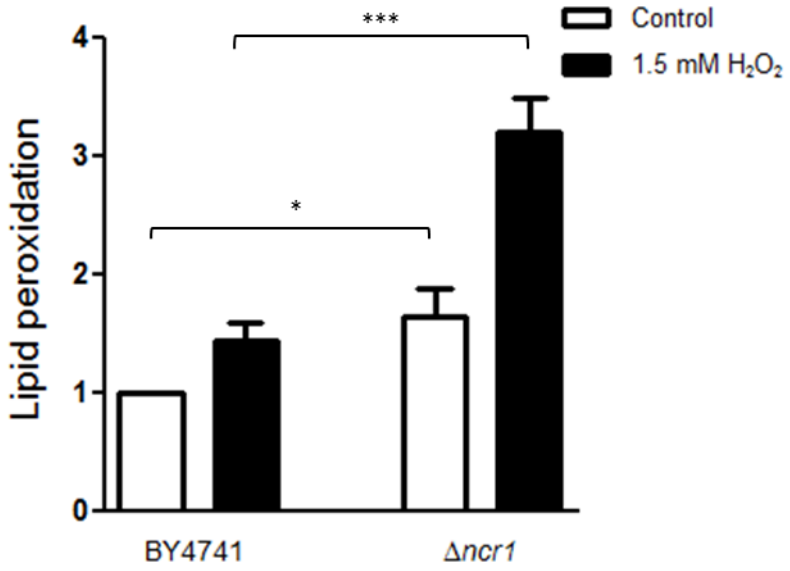


Figure 4.4 – Lipid peroxidation. *S. cerevisiae* BY4741 and Δ*ncr1* mutant cells were grown to exponential phase and treated with 1.5 mM H_2O_2 for 1h. Lipid peroxidation was quantified as described in methods. Data were normalized for untreated BY4741 cells. Values are mean ± SD of at least three independent experiments. Values were compared by Student´s t-test.*, $p< 0.05$; ***, $p< 0.001$

2.3. Protein carbonylation

Protein oxidation can be induced by ROS and products of lipid peroxidation and include the oxidation of aminoacids and cofactors. The aminoacids more prone to oxidation are tryptophan, tyrosine, lysine, arginine, proline, histidine, methionine and cysteine [55]. The protein carbonyl content has been widely used as a biomarker of

oxidative stress mainly because of its early formation and the relative stability of carbonylated proteins [99].

To evaluate protein oxidation, *S. cerevisiae* BY4741 and Δ*ncr1* mutant cells were treated with 1.5 mM H_2O_2, and protein extracts were slot-blotted to a PVDF membrane or resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The immunodetection of protein carbonyls showed a 4-fold increase in parental cells exposed to H_2O_2 (Figure 4.5). Ncr1p deficiency significantly increased H_2O_2-induced protein oxidation: after exposure to this oxidant, carbonyl content increased 7-fold in Δ*ncr1* mutant cells. In addition, the basal levels of protein carbonylation were 2-fold higher in Δ*ncr1* cells, when compared to parental cells. These results suggest that the sensitivity of Δ*ncr1* mutant cells to H_2O_2 is also associated with higher levels of protein carbonylation.

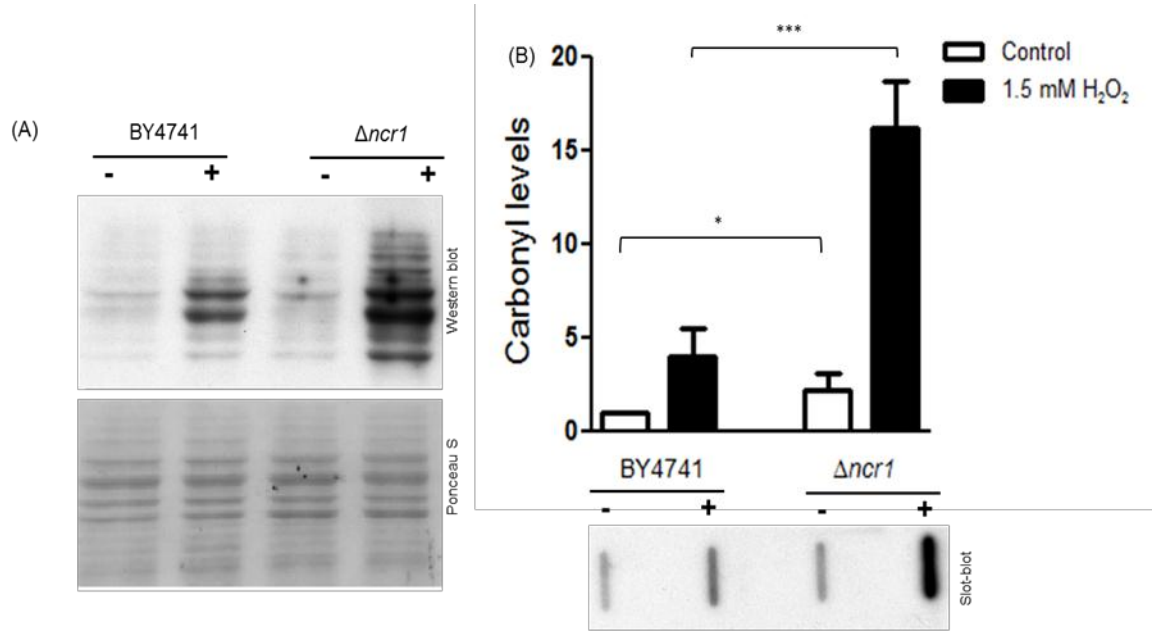


Figure 4.5 –Protein carbonylation. *S. cerevisiae* BY4741 and Δ*ncr1* cells were grown to exponential phase and treated with1.5 mM H_2O_2 for 1 h. Protein extracts were derivatized with DNPH and **(A)** – separated by SDS-PAGE, blotted into a nitrocellulose membrane and stained with Ponceau S (loading control). **(B)** – Slot-blotted into a PVDF membrane. Immunodetection was performed using an anti-DNP antibody, as described in methods. The quantification of the slot-blot bands was performed by densitometry. Data were normalized for untreated BY4741. Values are mean ± SD of at least three independent experiments. Values were compared by Student´s t-test.*, $p< 0.05$; ***, $p< 0.001$.

3. Menadione resistance

To evaluate if the oxidative stress sensitivity of Δ*ncr1* mutant cells was specific for hydrogen peroxide, cellular resistance to menadione, a superoxide generating drug, was also investigated. Menadione is a quinone that has been used as an agent for therapy of diseases like cancer. It is reduced by several enzymes, including microsomal NADPH-cytochrome P-450 redutase or mitochondrial NADH ubiquinone oxidoredutase, producing semiquinone radicals. Under aerobic conditions, these radicals participate in redox cycling to generate superoxide radicals [100].

To study menadione resistance, both parental and Δ*ncr1* mutant cells were treated for 1 hour with three different concentrations of this oxidant agent. The results show that menadione resistance of Δ*ncr1* mutant cells was similar to the observed in parental cells (Figure 4.6). This suggests that Δ*ncr1* mutant cells are specifically more sensitive to H_2O_2.

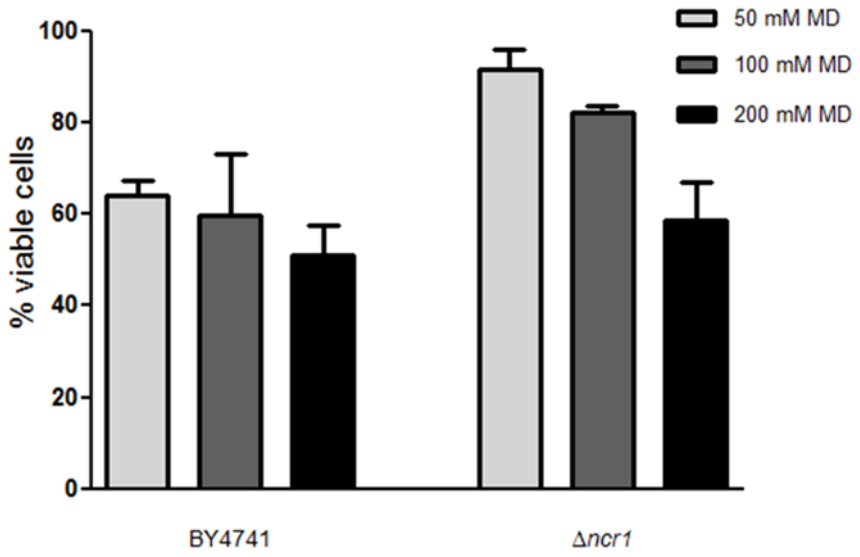


Figure 4.6 - Effect of menadione in oxidative stress resistance. *S. cerevisiae* BY4741 and Δ*ncr1* cells were grown to exponential phase and exposed to 50, 100 and 200 mM of menadione (MD) for 1 h. Cell viability was expressed as the percentage of the colony-forming units (treated cells vs. untreated cells). Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s t-test.

4. Mitochondrial dysfunction

Mitochondria are important for oxidative stress resistance. Previous work performed in our laboratory suggest that Ncr1p deficiency leads to mitochondrial dysfunction, as shown by the incapacity of these cells to grow in non-fermentable carbon sources, like glycerol, the decrease of cytochrome c oxidase activity and the destabilization of the mitochondrial network (Vilaça *et al.*, unpublished). Several proteins such as Tor1p, Sit4p and Sch9p have been implicated in the regulation of mitochondrial function. The absence of these proteins increases mitochondrial respiration and decreases the production of ROS whereas their activation has opposite effects [83].

To test if the activation of one of these proteins signalling pathway could be associated with the mitochondrial dysfunction observed in cells lacking Ncr1p, Δ*ncr1*Δ*tor1, Δncr1*Δ*sit4, and* Δ*ncr1*Δ*sch9* double mutants were constructed. *TOR1* was disrupted by homologous recombination, using a deletion fragment containing *KanMX4* and the flanking regions of this gene (Fig. 4.7) and the Δ*ncr1*Δ*tor1* mutants were selected in rich medium (YPD) containing geneticin. Gene deletion was confirmed by PCR. The Δ*ncr1*Δ*sit4* and Δ*ncr1*Δ*sch9* double mutant were constructed deleting *NCR1* in Δ*sit4* and Δ*sch9* cells. Gene disruption was also performed by homologous recombination, using a deletion fragment containing *URA3* and flanking regions of *NCR1*. The double mutants were selected in minimal medium without uracil.

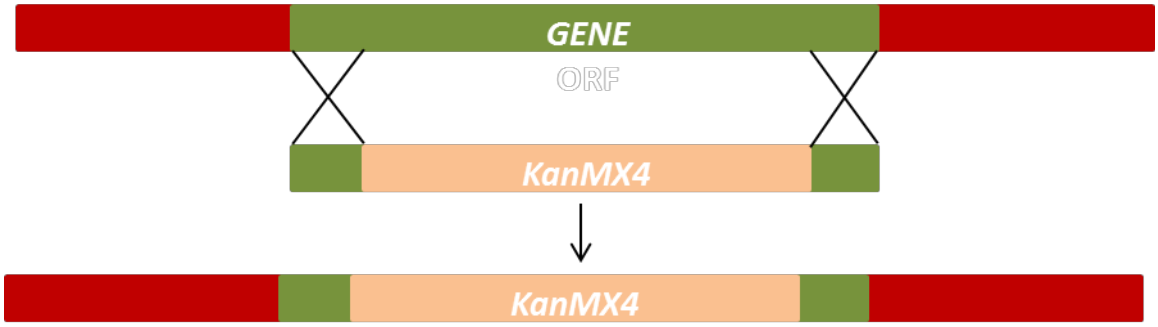


Figure 4.7 – Schematic representation of gene disruption by homologous recombination.

The deletion of *TOR1* was confirmed by PCR, using genomic DNA isolated from putative Δ*ncr1*Δ*tor1* double mutants or from BY4741 (negative control) and Δ*tor1* (positive control) cells, and the following primers: (1) *TOR1*_conf_Fw and Int*TOR1*_conf_Rv

(amplifies a fragment of 1011 bps using gDNA from cells containing *TOR1*); (2) *TOR1*_conf_Fw and Int*KanMX4*_conf_Rv (amplifies a fragment of 1500 bps using gDNA from cells deleted for *TOR1*). The *TOR1* gene was confirmed in BY4741 cells (set of primers 1), as expected, but not in Δ*tor1* cells or in the Δ*ncr1*Δ*tor1* double mutants. The results using the set of primers 2 confirmed the deletion of *TOR1* in the Δ*ncr1*Δ*tor1* double mutants and in Δ*tor1* cells (positive control) (Figure 4.8).

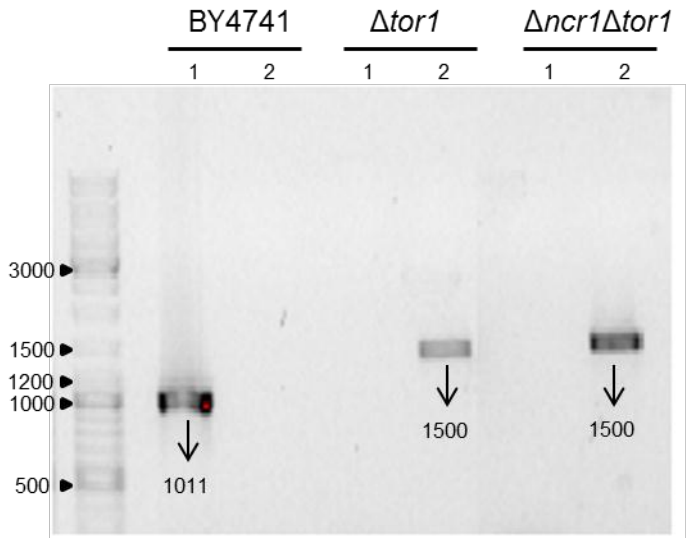


Figure 4.8 – Confirmation by PCR of *TOR1* deletion. *S. cerevisiae* BY4741 and Δ*tor1* cells, as well as Δ*ncr1*Δ*tor1* cells were grown to stationary phase, genomic DNA was extracted and PCR was performed as described in methods, using the following primers: 1- *TOR1*_conf_Fw + Int*TOR1*_conf_Rv; 2 – *TOR1*_conf_Fw + Int*KanMX4*_conf_Rv. The PCR products were analysed by electrophoresis using a 0.8% agarose gel. DNA bands were compared to Gene Ruler Ladder Mix.

A similar strategy was followed to confirm *NCR1* deletion, using genomic DNA isolated from putative Δ*ncr1*Δ*sit4* or Δ*ncr1*Δ*sch9* double mutants or from BY4741 (negative control) and Δ*ncr1* (positive control) cells, and the following primers: (3) *NCR1*_conf_Fw and Int*NCR1*_conf_Rv (amplifies a fragment of 1323 bps using gDNA from cells containing *NCR1*); (4) *NCR1*_conf_Fw and Int*URA3*_conf_Rv (amplifies a fragment of 1320 bps using gDNA from cells deleted for *NCR1*). The *NCR1* gene was confirmed in BY4741 cells (set of primers 3), as expected, but not in Δ*ncr1* cell or in

Δ*ncr1*Δ*sit4* and Δ*ncr1*Δ*sch9* double mutants. The results using the set of primers 4 confirmed the deletion of *NCR1* in the both double mutants (Δ*ncr1*Δ*sit4* and Δ*ncr1*Δ*sch9* cells) and in the positive control cells (Δ*ncr1*) (Figure 4.9.A and B).

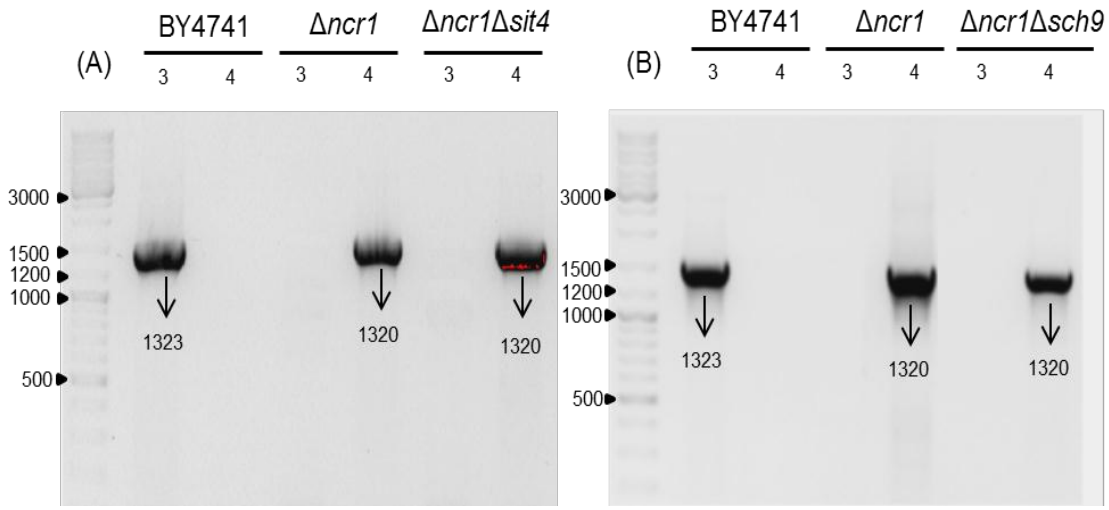


Figure 4.9 – Confirmation by PCR of *NCR1* deletion in (A) – Δ*sit4* cells and (B) – Δ*sch9* cells. *S. cerevisiae* BY4741, Δ*sit4* and Δ*sch9* cells, as well Δ*ncr1*Δ*sit4* cells and Δ*ncr1*Δ*sch9* cells were grown to stationary phase, genomic DNA was extracted and PCR was performed as described in methods, using the following primers: 3 - *NCR1*_conf_Fw + Int*NCR1*_conf_Rv; 4 – *NCR1*_conf_Fw+ Int*URA3*_conf_Rv. The PCR products were analysed by electrophoresis using a 0.8% agarose gel. DNA bands were compared to Gene Ruler Ladder Mix.

4.1. Inactivation of *SIT4 or SCH9*, in contrast with *TOR1* gene, supresses H_2O_2 sensitivity and growth defects on glycerol displayed by Δ*ncr1* cells

To test if deletion of *TOR1*, *SIT4 or SCH9* suppresses mitochondrial dysfunction phenotype associated to Ncr1p deficiency, parental cells, Δ*ncr1*, Δ*tor1*, Δ*sit4* and Δ*sch9* single mutants, and Δ*ncr1*Δ*tor1,* Δ*ncr1*Δ*sit4* and Δ*ncr1*Δ*sch9* double mutant cells were grown in SC-glucose medium to exponential phase, diluted to an $O.D._{600nm}$=0.1 and plated in solid SC medium containing glucose or glycerol as carbon source. Both parental cells (BY4741), Δ*tor1*, Δ*sit4* and Δ*sch9* mutant cells were able to grow in the presence of glucose or glycerol. As expected, Δ*ncr1* mutant cells were not able to grow in SC-glycerol (Figures 4.10A, 4.11A and 4.13A). This phenotype was not reverted by *TOR1* deletion. Moreover, Δ*ncr1*Δ*tor1* cells displayed a high sensitivity to H_2O_2, as observed in Δ*ncr1* single mutants (Figure 4.10B). These results suggest that the TORC1 pathway is not associated with the low resistance of Δ*ncr1* mutant cells to H_2O_2.

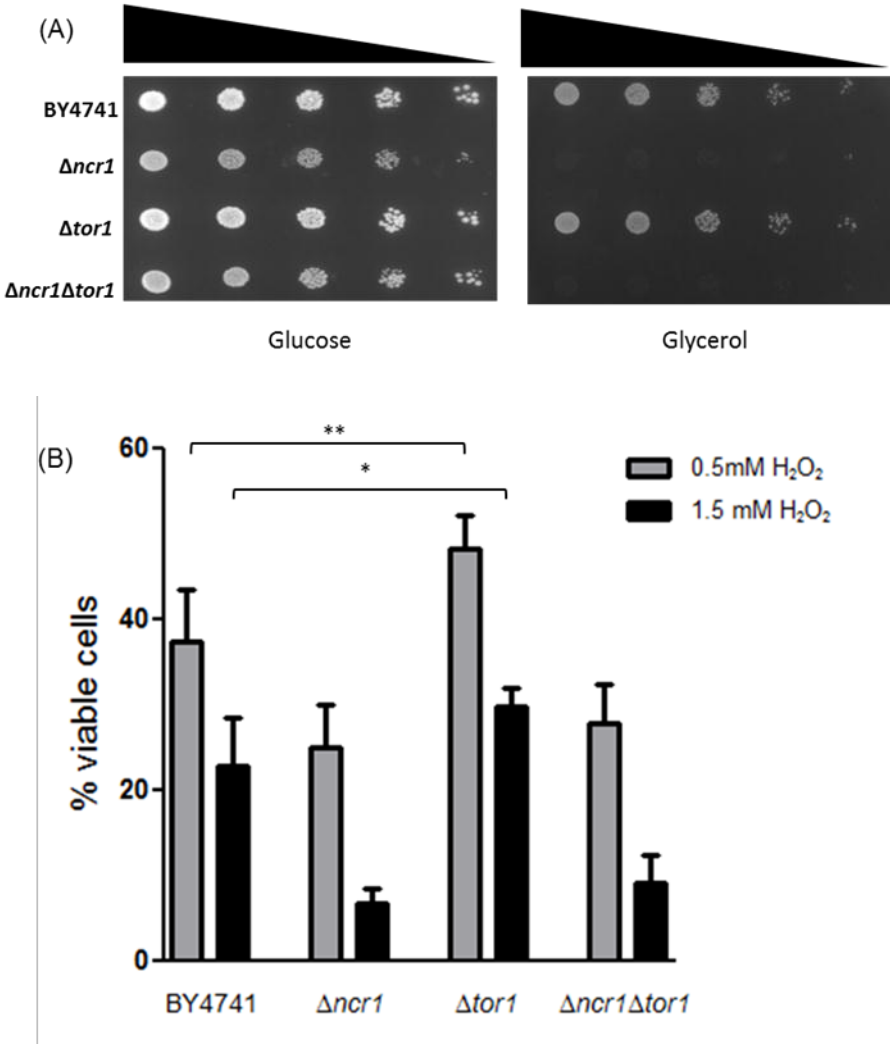


Figure 4.10 – Deletion of *TOR1* does not suppress Δ*ncr1* phenotypes. (A) – *S. cerevisiae* BY4741, Δ*ncr1*, Δ*tor1* and Δ*ncr1*Δ*tor1* cells were grown to exponential phase, diluted to a $O.D_{600}$=0,1 and fivefold serial dilutions were plated in SC-medium containing glucose or glycerol as carbon source. **(B)** – Yeast cells were grown to exponential phase and exposed to 0.5 and 1.5 mM H_2O_2 for 1 h. Cell viability was expressed as the percentage of the colony-forming units (treated cells vs. untreated cells). Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s t-test.*, $p< 0.05$; **, $p< 0.01$;

In contrast, the deletion of *SIT4* suppressed the respiratory defects and H_2O_2 sensitivity of Δ*ncr1* mutant cells (Figure 4.11). Consistent with published data, Δ*sit4* mutant cells presented a higher resistance to this drug, compared to the observed in parental cells (Figure 4.11B). These results suggest that the pathway involving Sit4p is associated with Δ*ncr1* mutant cells phenotype.

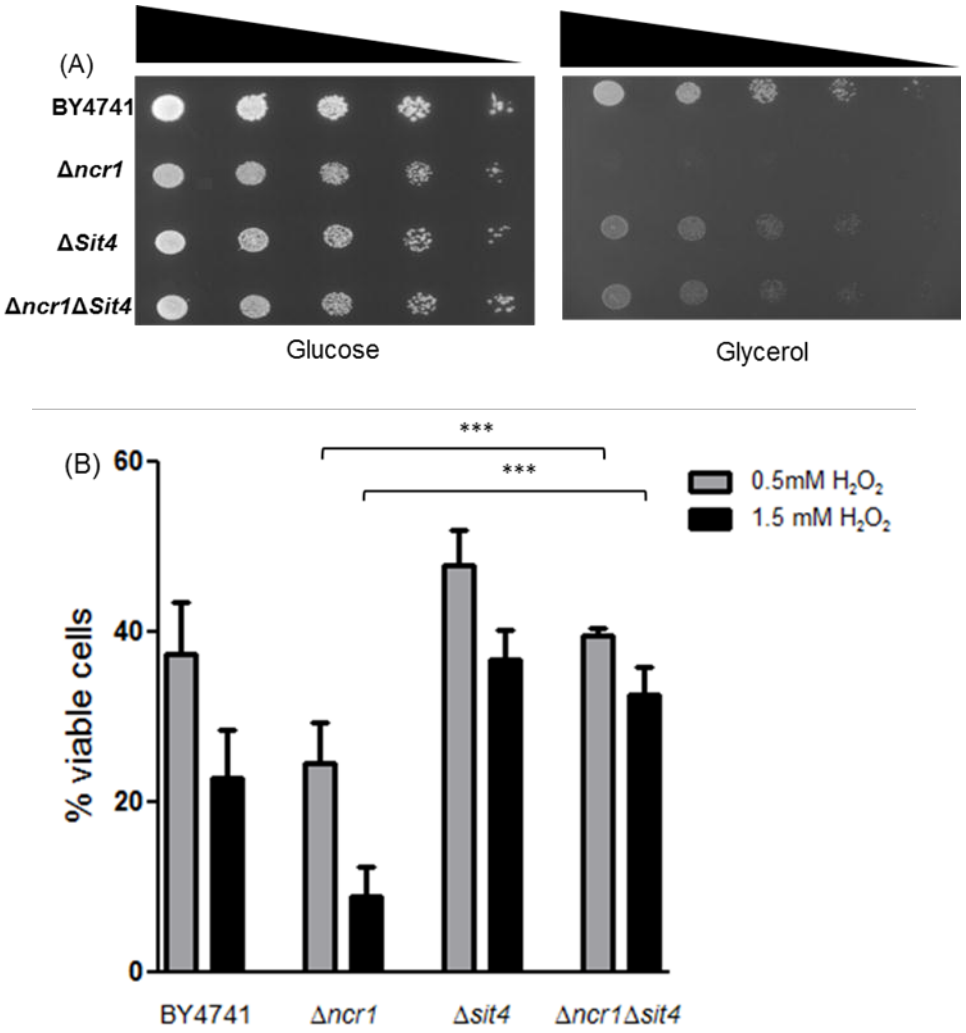


Figure 4.11 – Deletion of *SIT4* suppresses Δ*ncr1* phenotypes. (A) – *S. cerevisiae* BY4741, Δ*ncr1* , Δ*sit4* and Δ*ncr1*Δ*sit4* cells were grown to exponential phase, diluted to a $O.D_{600}$=0,1 and fivefold serial dilutions were plated in SC-medium containing glucose or glycerol as carbon source. **(B)** –Yeast cells were grown to exponential phase and exposed to 0.5 and 1.5 mM H_2O_2 for 1 h. Cell viability was expressed as the percentage of the colony-forming units (treated cells vs. untreated cells). Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s t-test. ***, p< 0.001

To test if the increase in the resistance of the Δ*ncr1*Δ*sit4* double mutant cells to H_2O_2 was associated with lower levels of intracellular ROS, Δ*sit4* and Δ*ncr1*Δ*sit4* mutant cells were treated with 1.5 mM H_2O_2 for 50 minutes and subsequently labelled with DHE. The results showed an increase of DHE-positive cells induced by H_2O_2 that was similar in both Δ*sit4* and Δ*ncr1*Δ*sit4* mutant cells (Figure 4.12A). However, this increase was significantly lower to the observed in Δ*ncr1*, which is consistent with the higher H_2O_2 resistance exhibited by Δsit4 and *Δncr1Δsit4*. This result further supports the hypothesis that Sit4p is involved in mitochondrial dysfunction associated with Ncr1p deficiency.

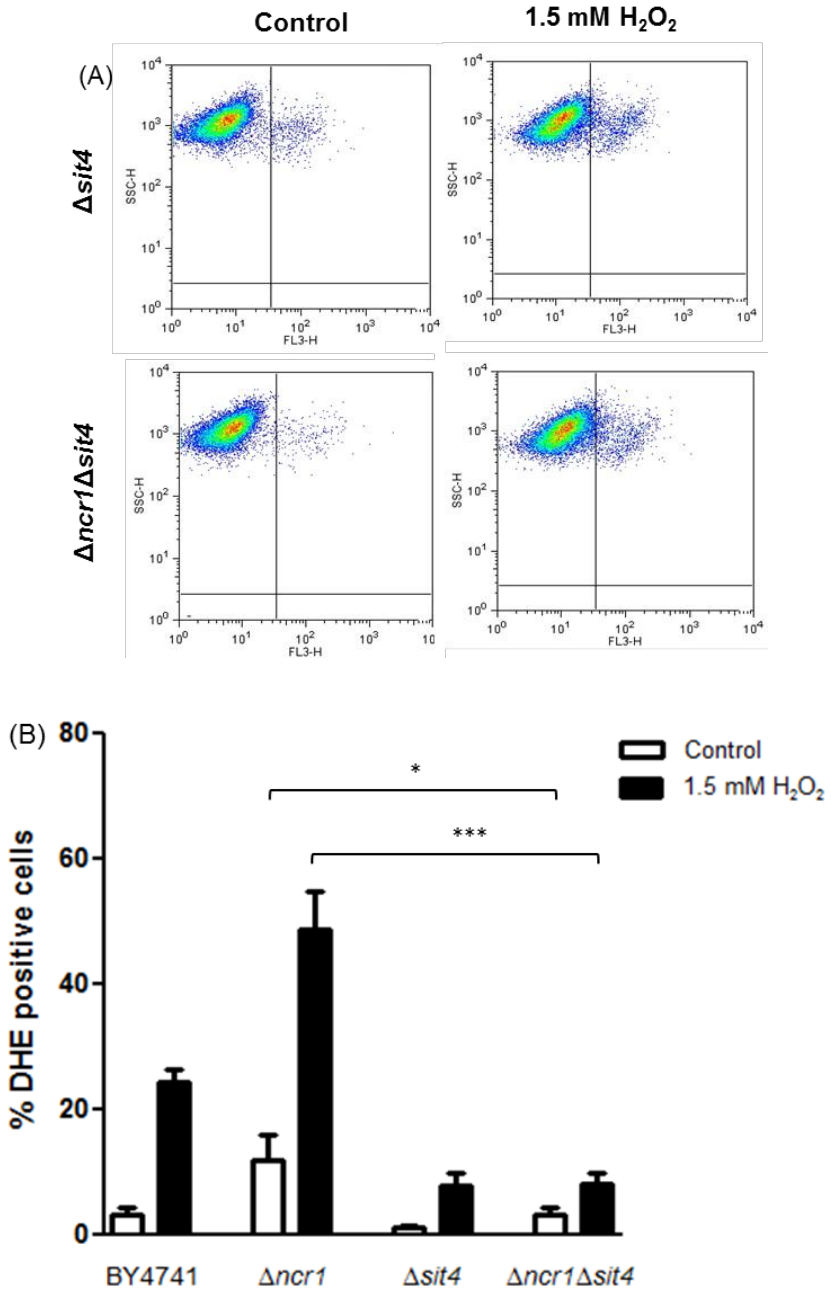
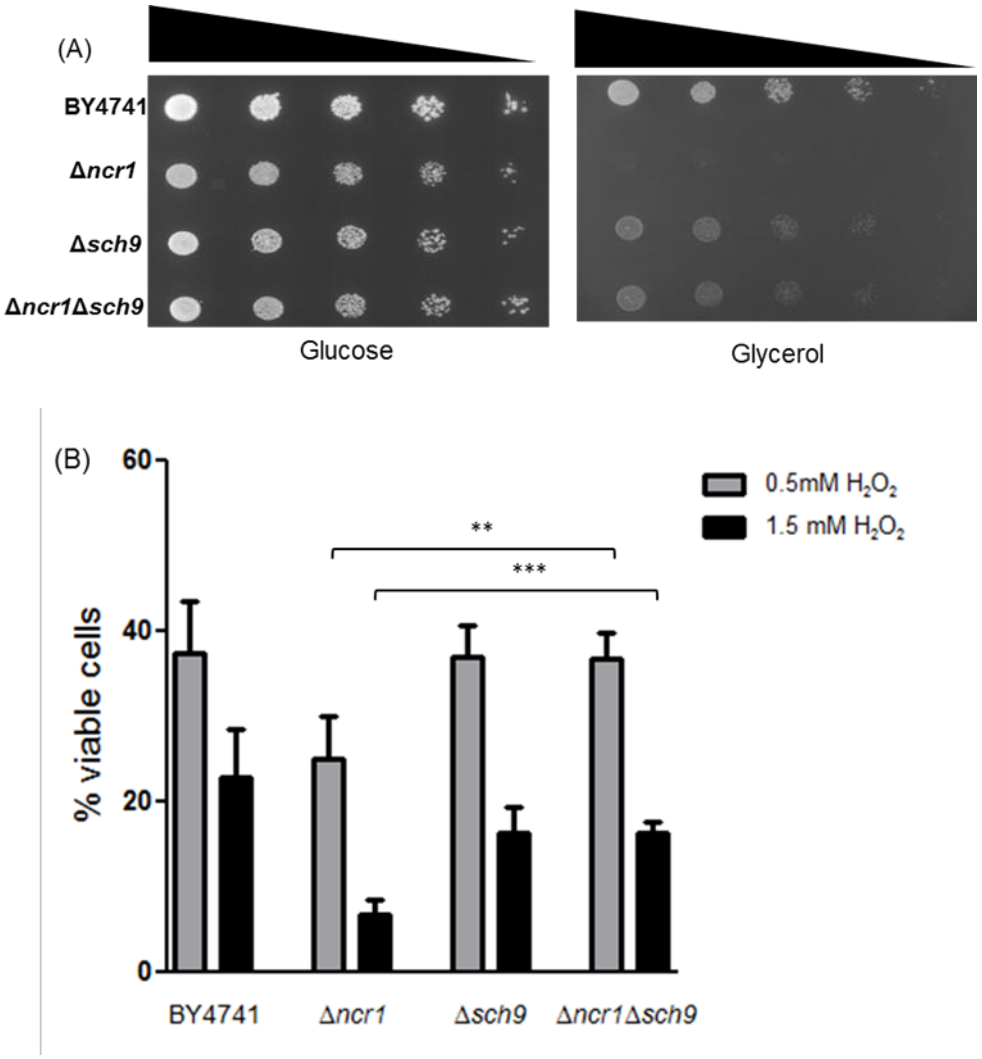


Figure 4.12 - Quantification of intracellular ROS. *S. cerevisiae* BY4741, Δ*ncr1,* Δ*sit4* and Δ*ncr1*Δ*sit4* cells were grown to exponential phase, treated with 1.5 mM of H2O2 for 50 minutes labelled with DHE for 10 minutes and analysed by FACS as described in methods. (A) – FACS plot analysis of cells untreated and treated with H2O2 (B) – Quantification of the intracellular ROS levels using information from FACS plots. The % of positive cells was quantified by Flowjo software analyses. The values for BY4741 and Δ*ncr1* cells are from Fig 4.3. Data are mean ± SD of at least three independent experiments. Values were compared by Student´s t-test.*, *p*< 0,05;***, p< 0.001

In the same way, the deletion of *SCH9* suppressed the respiratory defects and H_2O_2 sensitivity of Δ*ncr1* mutant cells (Figure 4.13). As expected Δ*sch9* mutant cells

presented a lower sensitivity to this drug, compared to the observed in parental cells (Figure 4.13B). These results suggest that the pathway involving Sch9p is also associated with Δ*ncr1* mutant cells phenotype.



Figures 4.13 – Deletion of SCH9 suppress Δ*ncr1* phenotypes. (A) – *S. cerevisiae* BY4741, Δ*ncr1*, Δ*sch9* and Δ*ncr1*Δ*sit4* cells were grown to exponential phase, diluted to a $O.D_{600}$=0,1 and fivefold serial dilutions were plated in SC-medium containing glucose or glycerol as carbon source. **(B)** –Yeast cells were grown to exponential phase and exposed to 0.5 and 1.5 mM H_2O_2 for 1 h. Cell viability was expressed as the percentage of the colony-forming units (treated cells vs. untreated cells). Data were expressed as mean values ± SD of at least three independent experiments. Values were compared by Student´s t-test. **, p< 0.01; ***, p< 0.001

This increase in the resistance of the Δ*ncr1*Δ*sch9* double mutant cells to H_2O_2 was associated with lower levels of intracellular ROS. The results showed an increase of DHE-positive cells induced by H_2O_2 that was similar in both Δ*sch9* and Δ*ncr1*Δ*sch9* mutant cells but significantly lower to the observed in parental or Δ*ncr1* cells (Figure 4.14). This

supports the hypothesis that Sch9p is involved in mitochondrial dysfunction associated with Ncr1p deficiency.

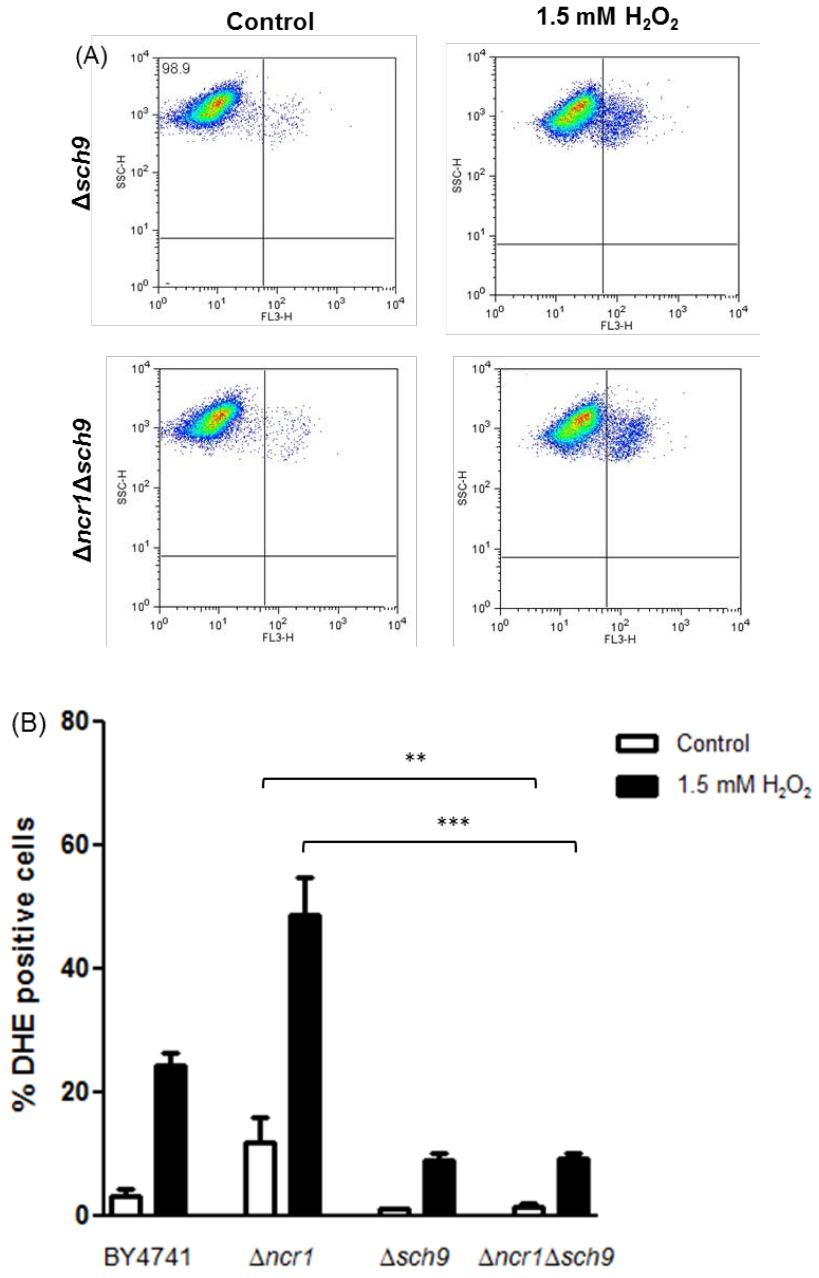


Figure 4.14 - Quantification of intracellular ROS. *S. cerevisiae* BY4741, Δ*ncr1,* Δ*sch9* and Δ*ncr1*Δ*sch9* cells were grown to exponential phase, treated with 1.5 mM of H_2O_2 for 50 minutes labelled with DHE for 10 minutes and analysed by FACS as described in methods. (A) – FACS plot analysis of cells untreated and treated with H_2O_2 (B) – Quantification of the intracellular ROS levels using information from FACS plots. The % of positive cells was quantified by Flowjo software analyses. The values for BY4741 and Δncr1 cells are from Fig 4.3. Data are mean ± SD of at least three independent experiments. Values were by Student´s t-test. **, p< 0.01; ***, p< 0.001

Chapter 5

Discussion

Discussion

Sphingolipid storage diseases have been a main topic of research in the last years. The molecular pathology of these disorders has been subject of study in an attempt to understand the processes underlying these diseases.

Niemann-Pick type C is an autosomal recessive lipid storage disease characterized by hepatosplenomegaly and severe progressive neurological dysfunction [2]. The clinical spectrum of NPC is extremely heterogeneous, and at the cellular level it is characterized by unique abnormalities in intracellular transport and accumulation of endocytosed cholesterol in lysosomes or late endosomes [28]. It can be caused by mutations in two different proteins, namely NPC1 and NPC2 that seem to mediate the proper intracellular lipid transport through pathways that remain unclear [1].

Reactive oxygen species are normal by-products of aerobic metabolism and are mainly produced by leakage of electrons in the mitochondrial electron transport chain [48]. Accumulation of ROS due to perturbation of control mechanisms can result in the accumulation of mutations, premature ageing and cell death. Excess of oxidative stress is implicated in several neurodegenerative disorders including NPC [45,46]. Studies using different NPC cellular models [60], as well as in mice tissues [101] including $NPC^{-/-}$ mice brain [63] suggest that oxidative stress as an important role in the pathophysiology of Niemann-pick type C disease. However, the role of oxidative stress in this and other neurodegenerative disorders [46] is still unclear, and it is still unknown if changes in redox homeostasis are cause or consequence of neurons degeneration [102].

In this work, we investigated the role of Ncr1p, the yeast orthologue of human NPC1 protein [71], in oxidative stress resistance. Our results show that cells lacking Ncr1p were more sensitive to oxidative stress induced by H_2O_2, but not by menadione, a superoxide generating drug. This phenotype was not strain specific and was partially rescued by *NCR1* expression, indicating that it is related to Ncr1p deficiency.

Consistent with the hypersensitivity to H_2O_2, Δ*ncr1* mutant cells showed higher levels of oxidative stress markers, such as ROS, lipid peroxidation and protein carbonylation. Other studies performed in our laboratory showed that Ncr1p deficiency leads to a significant decrease in the levels of glutathione and in the activity of cytosolic catalase and mitochondrial superoxide dismutase (Vilaça *et al.*, unpublished). Lower levels of antioxidant defences were also described in different NPC models, including a decrease of glutathione content in NPC mice livers [101] and of catalase activity in fibroblasts from NPC patients and in experimentally-induced NPC1 knockdown in primary cultures of fibroblasts or human SHSY5Y neuroblastoma cells [60]. Thus, the decrease in

antioxidant defences may contribute to an increase in ROS levels and accumulation of oxidized molecules. Lipids exhibit a high susceptibility to oxidation, generating reactive products that will extend the pro-oxidant effects of ROS by damaging DNA and proteins [54]. Damaged proteins that cannot be repaired must be targeted to proteolytic systems to prevent the accumulation of proteins aggregates. Failure in oxidised proteins clearance can contribute to mitochondrial dysfunctions, further increasing ROS production [50,55].

We hypothesize that the increase of intracellular oxidation in cells lacking Ncr1p is related with mitochondrial dysfunction. Indeed, our lab showed that Δ*ncr1* mutant cells are unable to grow in non-fermentable carbon sources, which require functional mitochondria, and display mitochondrial fragmentation and destabilization of mitochondrial membrane potential (Vilaça et *al.*, unpublished). Some authors have demonstrated impaired mitochondrial function in other NPC models. $NPC1^{-/-}$ mice exhibit a decrease in mitochondrial membrane potential associated with a decrease in the synthesis of ATP. This is due to an increase in the concentration of cholesterol in the mitochondrial membrane, altering its fluidity [65]. Mari and co-workers have reported a decrease of mitochondrial GSH in NPC hepatocytes while the cytosolic GSH remains unaltered [103]. The increase in mitochondrial cholesterol seems to influence the transport of GSH to the mitochondria, since it depends on the membrane fluidity [45]. Another study showed an increase in ubiquinone-10 (oxidized form) and the respective decrease in ubiquiol-10 (reduced form), an imbalance that suggests mitochondrial dysfunctions [62].

A process that is associated to oxidative stress is ageing. “The Free Radical Theory of Ageing” links mitochondrial dysfunction to overproduction of ROS, causing damage in the cells that leads to premature ageing [47]. Studies in our lab showed that Δ*ncr1* mutant cells exhibit a shortened chronological lifespan (Vilaça *et al.*, unpublished). The oxidative stress changes described in almost all NPC models suggest that the imbalance in the intracellular redox state may not be restricted to a single cell type, but is a general event in NPC phenotype.

The mechanisms involved in the regulation of mitochondrial function are complex. In yeast, cell signalling mediated by the Tor1p and Sch9p protein kinases and by the Sit4p protein phosphatase has been shown to play a key role in mitochondrial regulation (see Figure 1.7).

The Tor1p is a serine-threonine protein kinase conserved in all eukaryotes. Tor1p resides in a multi-protein complex, TORC1 (sensitive to rapamycin). TORC1 is regulated by nutrients more specifically nitrogen source [77]. Sit4p is a serine-threonine Ceramide-activated protein phosphatase related to type 2A family of protein phosphatases (PP2A) that are involved in cell cycle regulation [84,85]. Sch9p is a serine-homologue to

mammalian ribosomal S6 kinase (S6K) [88] or Protein Kinase B (PKB/AKT) [89]. Sch9p is activated by glucose metabolism [90] and by sphingolipids such as phytosphingosine through Pkh1/2p pathway [80]. Down-regulation of these proteins promotes mitochondrial function, decreases oxidative stress and increases chronological lifespan [74,75,80]. Therefore, we postulated that the deregulation of these proteins could be implicated in the mitochondrial dysfunction and increased oxidative stress markers displayed by Ncr1p deficient cells. Interestingly, our results suggest that these phenotypes are Sit4p-dependent. In addition, although Sch9p is activated by Tor1p, *SCH9* deletion, in contrast with *TOR1* deletion, suppressed mitochondrial dysfunction and sensitivity to oxidative stress in cells lacking Ncr1p. These results suggest that changes in Sit4p- and Sch9p-dependent cell signalling in Δ*ncr1* mutant cells are Tor1p-independent. Since Sit4p is a ceramide-activated protein phosphatase [84] and Sch9p is activated by phytosphingosine through Pkh1p/2p [73], it is likely that changes in these bioactive sphingolipids over-activate these proteins Δ*ncr1* mutant cells. To test this hypothesis, a lipidomic analysis should be performed to determine the levels of the different bioactive sphingolipids in parental and Δ*ncr1* cells. In addition, studies to evaluate the activation of the Sit4p and Sch9p pathways should be performed. This may be executed through the analysis by western-blot of the levels of phosphorylated and active form of these proteins. Sphingosine is highly accumulated in all tissues of the body of NPC patients, increasing 4-fold in brains and 12-fold in livers [11]. In addition, the increase of sphingosine per se can promote NPC phenotype in normal cells [11]. Thus, we predict that the increase of phytosphingosine in Δ*ncr1* cells may explain the over-activation of Sch9p pathway that induces mitochondrial dysfunction.

The *Saccharomyces cerevisiae* used as a model for this study is of an extreme importance. The simplicity of the organism and the facility to manipulate it makes it easier to characterize cellular processes conserved during evolution, such as signalling pathways. The fact that Ncr1p, Tor1p, Sit4p and Sch9p have orthologues in mammalians, including humans, makes it possible to translate our results to higher organisms.

In summary, our results indicate that Ncr1p play an important role in oxidative stress resistance. Deletion of *SCH9* or *SIT4* suppresses mitochondrial dysfunction, decreasing intracellular oxidation and the sensitivity to H_2O_2 of Δ*ncr1* mutant cells. The mechanism underlying this reversion of phenotype is still unclear and has to be characterized. Future studies are required to assess if the signalling pathways identified in yeast are also affected in more complex models such as fibroblasts from NPC patients, and if the pharmacological manipulation of these pathways minimizes or delays the progression of NPC disease.

Chapter 6

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